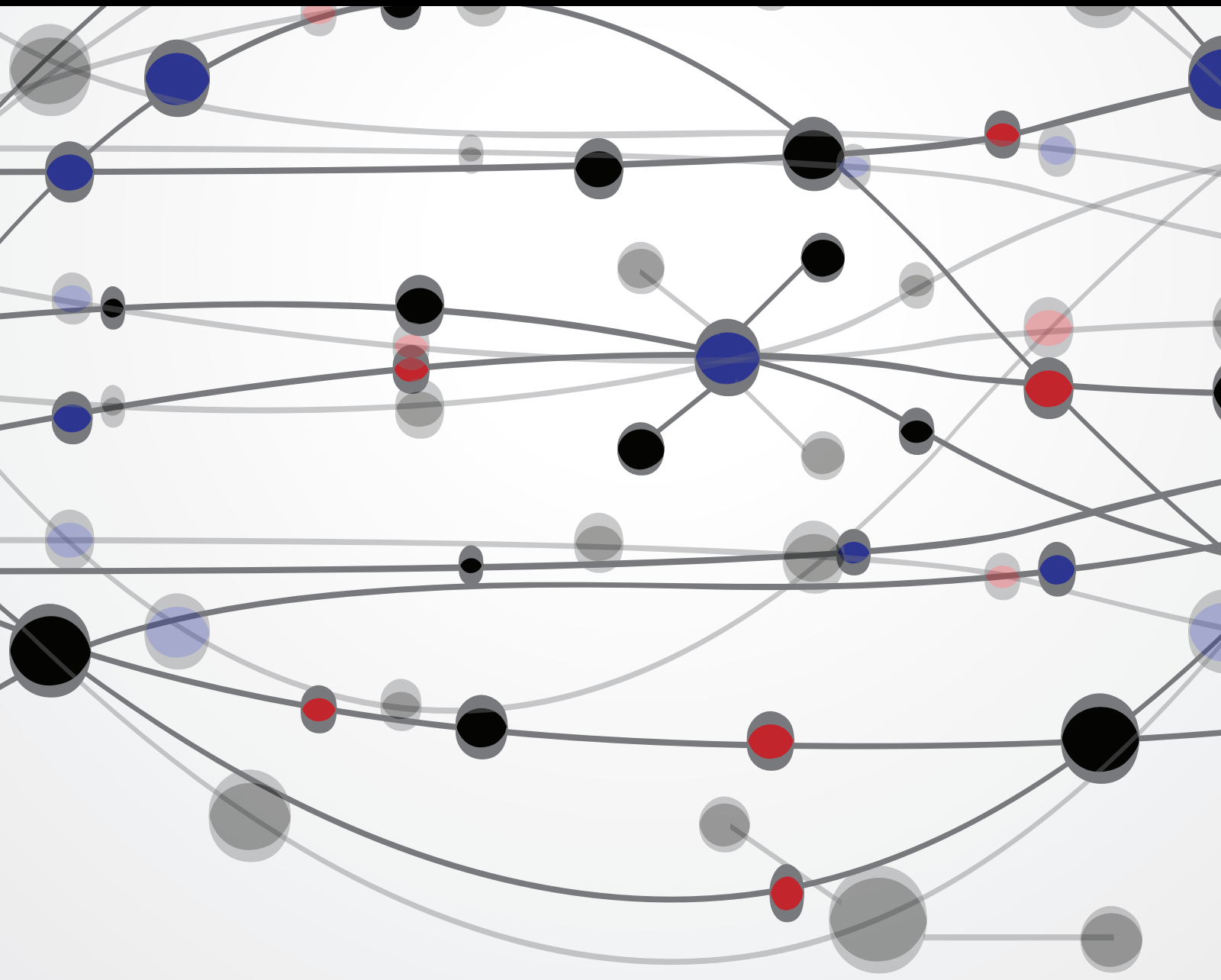
The Scientific World Journal

# Prostate Cancer: All Aspects

Guest Editors: Ahmet Tefekli, Murat Tunc, Volkan Tugcu, and Tarık Esen



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# Contents

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*Editorial*

# Prostate Cancer: All Aspects

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Prostate cancer is currently the most common cancer diagnosed in males living in developed nations and is becoming more commonly encountered in the rest of the world. This increase in the prevalence of prostate cancer is basically due to the advent of "Prostate Specific Antigen (PSA)" screening. However, an exciting debate and research are going around whether to screen or not, and more details are discussed in a review in this special issue. Diagnostic techniques have also evolved and have facilitated detection of majority of significant tumors. On the other hand, issues surrounding treatment are complex. Radical treatment options for prostate cancer are themselves not innocuous. However observing patients (active surveillance) carries its own risk of progression, especially because current methods of diagnosis do not distinguish aggressive from indolent tumors. Taking all these facts into consideration, it seems that much more effort will be spent on the research of prostate cancer in the near future. And therefore we decided to publish a special issue on prostate cancer, trying to cover all aspects of this important issue. Many papers especially from Asia and South America were submitted to this special issue and even only this demographic information is almost enough to underline the importance of prostate cancer as a growing health problem all over the world. And herein, outstanding findings of the published papers of this special issue are summarized.

In a timely interesting study from Austria by D. Junker et al., potentials and limitations of real-time elastography (RTE) for prostate cancer detection were assessed, considering the fact that cancers usually have a higher cell and vessel density than normal tissue and are therefore associated with a decreased elasticity. RTE findings were compared with whole-mount step section analysis of the prostate obtained from radical prostatectomy. RTE could detect 9.7% of cancer lesions with a maximum diameter of 0–5 mm, 27% of cancer lesions with a maximum diameter of 6–10 mm, and 70.6% of lesions with a maximum diameter of 11–20 mm while it could show 100% of lesions with a maximum diameter of >20 mm. In addition, there was a significant higher rate of cancer detection rate in those with predominant Gleason pattern 4 or 5 regarding cancer lesions with a volume $\geq 0.2\,cm^3$. And the authors further suggest that adding information about contrast media dynamics in a multiparametric way might decrease the number of false negative cases in the ultrasonic evaluation of prostate cancer.

Several preoperative and postoperative nomograms, most of them originating from western countries, are being used in order to predict the outcome of prostate cancer, and many validations studies have been published in the literature. In an interesting study by V. H. W. Yeung et al. from Hong Kong, predictive accuracy of Kattan and Stephenson nomograms in the Chinese population was investigated for the first time in the literature. Despite a limited number of patients, the authors could observe that the 5-year and 10-year biochemical free survival rates in Chinese patients were similar to the predicted values by the Kattan and Stephenson nomograms.

Almost 90% of prostate cancer cases diagnosed in the era of PSA are believed to be "organ confined," and "radical prostatectomy (open or laparoscopic/robot assisted)" is conserved to be the treatment of choice in the majority of cases. There have been a tremendous number of publications in the last decade about laparoscopic radical prostatectomy, investigating the outcome, learning curve, and almost all other aspects. But probably nobody has ever dared to analyze

or publish the learning curve of a low volume surgeon. A. I. Mitre et al. from Brazil investigated this common but "never asked" question. The authors conducted a prospective study on 165 patients operated over an 8-year period by a single surgeon with previous laparoscopic experience. Sequential analyses were performed and, in order to define the learning curve, patients were divided into 3 groups of 55 patients arranged in chronological order. The results showed that intraoperative complications and conversions to open surgery were significantly less after the first 51 cases. All other parameters (blood loss, operative time, and positive surgical margins) significantly decreased and stabilized after 110 cases. Although there are several limitations in the study, the authors have to be congratulated for their efforts to collect and report their results.

Radical prostatectomy is nowadays the treatment of choice in the management of organ confined prostate cancer, and biochemical failure, reported to develop 20–30%, is becoming more commonly encountered. N. P. Murray et al. from Chile examined the presence of circulating prostate cells in blood after radical prostatectomy, using standard immunocytochemistry with anti-PSA monoclonal antibodies. They reported that circulating prostate cancer cells in blood were detected more frequently in patients with positive margins, capsular invasion, and vascular and lymphatic infiltration. They also concluded that presence of circulating prostate cancer cells was an independent risk factor associated with biochemical recurrence.

There are also several research studies published in this special issue. In a very interesting cell culture study submitted from Baltimore, USA, by A. Gupta et al., researchers examined the biological consequences of matrix metalloproteinase 9 (MMP9) knockdown in the invasion of prostate cancer (PC3) cells. It has been previously shown that MMP9 localized in invadopodia facilitates extracellular matrix degradation and invasion in PC3 human prostate carcinoma cells, by switching CD44 isoform expression from CD44 standard to CD44v6, which may be essential for the protection of noninvasive cellular phenotype. Although there are conflicting results regarding expression of CD44 and tumor characteristics, the researchers were the first to show that MMP9 knockdown increased CD44v6 expression and suggest that interaction between CD44 and MMP9 is a potential mechanism of invadopodia formation in PC3 cells. They also postulate that CD44v6 may be a potential marker for prognosis.

In another cell line study (LNCaP cell line) by S. S. Kim et al. from Korea, factors related to the development of androgen independent prostate cancer were investigated. High passage subcultured LNCaP cells acquired androgen independent property and the silencing of androgen receptor (AR) with small interfering RNA (siRNA) transfection resulted in the reversion of proteomic profile to level of fresh cell line. Furthermore, the expressions of five cancer related proteins (AR, heat-shock protein 27, clusterin, glucose-related protein 78, and cellular FLICE-like inhibitory protein) were increased in late stage (over 81 times subcultured LNCaP cell line) LNCaP. However these cancer related protein expressions were reversed with small interfering RNA (siRNA) transfection. These findings support that therapeutic approaches targeting AR can enhance the efficacy of anticancer treatment in the patients with castration resistant prostate cancer.

In another research study using PC3 cell line, S. S. Kim et al. from Korea investigated the change of doxazosin induced apoptosis after dual gene silencing of heat-shock protein 27 and cellular FLICE-like inhibitory protein (c-FLIP) in PC-3 cancer cells. They elegantly showed that dual silencing using siRNAsis technically feasible and knock out of c-FLIP and Hsp27 gene together enhances apoptosis with doxazosin in PC-3 cells. This finding suggests a new strategy of multiple knockout of antiapoptotic and survival factors in the treatment of late stage prostate cancer refractory to conventional therapies.

And finally, the guest editors of this special issue summarized the future prospects in the management of localized prostate cancer. As a conclusion, prostate cancer is a wide fertile area for both basic and clinical research and revolutionary changes in the diagnosis and management of prostate cancer are awaited in the near future.

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*Review Article*

# Future Prospects in the Diagnosis and Management of Localized Prostate Cancer

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Prostate cancer (PCa) is the commonest visceral cancer in men worldwide. Introduction of serum PSA as a highly specific biomarker for prostatic diseases has led to a dramatic increase in the diagnosis of early stage PCa in last decades. Guidelines underline that benefits as well as risks and squeals of early diagnosis and treatment should be discussed with patients. There are several new biomarkers (Pro-PSA, PCA-3 test, and TMPRSS2-ERG) available on the market but new ones are awaited in order to improve specificity and sensitivity. Investigators have also focused on identifying and isolating the gene, or genes, responsible for PCa. Current definitive treatment options for clinically localized PCa with functional and oncological success rates up to 95% include surgery (radical prostatectomy), external-beam radiation therapy, and interstitial radiation therapy (brachytherapy). Potential complications of overdiagnosis and overtreatment have resulted in arguments about screening and introduced a new management approach called "active surveillance." Improvements in diagnostic techniques, especially multiparametric magnetic resonance imaging, significantly ameliorated the accuracy of tumor localization and local staging. These advances will further support focal therapies as emerging treatment alternatives for localized PCa. As a conclusion, revolutionary changes in the diagnosis and management of PCa are awaited in the near future.

## 1. Introduction

Prostate cancer (Pca) is the most common noncutaneous malignancy and the second leading cause of cancer death in men [1]. According to very recently published statics, cancers of the prostate, lung and bronchus, and colorectum will account for about half of all newly diagnosed cancers among men while prostate cancer alone is underlined to account for 29% (241,740) of incident cases [1]. Furthermore, cancers of the lung and bronchus, prostate, and colorectum in men will continue to be the most common causes of cancer death [1].

In the United States, 90% of men with Pca are older than 60 years, diagnosed by early detection with the serum prostate-specific antigen (PSA) blood test, and have disease believed to be confined to the prostate gland [2]. Considering these factors as well as the sociocultural position of this group of men, the treatment of the localized Pca stands out as a major health problem.

Current treatment options for clinically localized Pca include active surveillance (AS), surgery (radical prostatectomy), external-beam radiation therapy, and interstitial radiation therapy (brachytherapy) [3]. Highly satisfactory success rates up to 95% are being reported using a single or a combination of these treatment modalities [3].

## 2. Screening and Early Detection

Improved treatment techniques as well as earlier diagnosis in recent years have certainly led to better results [3]. However, early diagnosis and/or early treatment of Pca has interestingly not improved the Pca specific survival or overall survival from Pca [4]. On the other hand, there is an everyday increasing number of publications dealing with new markers to detect Pca in the early stage [5]. Although PSA is a prostate specific marker, it is generally agreed that the PSA test is not a perfect test for finding Pca in its early phase. In order to improve

the sensitivity and specificity of serum PSA, several PSA derivates and isoforms are being used [5].

Prostate Health Index (Phi index: *Phi index: [−2] proPSA/fPSA* × $PSA^{1/2}$) has recently been suggested as a useful tool by Catalona et al., especially in men with a serum PSA 2–10 ng/mL [6]. Previous studies have shown that elevated (pro-PSA/free PSA) ratios are associated with aggressive pathological features and decreased biochemical disease-free survival after radical prostatectomy [7]. A new automated tool using the [−2]proPSA assay with a percent-free PSA-based artificial neural network was reported to be capable of detecting Pca and more aggressive diseases with higher accuracy than total PSA or percent-free PSA alone [8]. In a recent prospective cohort of men enrolled into active surveillance for PCa, serum and tissue levels of pro-PSA at diagnosis were associated with the need for subsequent treatment [9]. The authors suggested that the increase in the ratio of serum pro-PSA to percent-free PSA might be driven by increased pro-PSA production from "premalignant" cells.

Despite the worldwide popularity of PSA, there are still debates going around it [10]. First of all, it is questioned whether PSA-based screening decreases prostate cancer-specific or all-cause mortality [3, 11]. In the recently published PIVOT study which was performed among men with localized Pca detected during the early era of PSA testing, radical prostatectomy did not significantly reduce all-cause or Pca mortality, as compared with observation, through at least 12 years of followup [11]. Furthermore, in another recently published prospectively randomized study called the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer screening Trial, it was also concluded that Pca mortality was not significantly different between the PSA (and DRE) screened and control groups [12]. On the other hand, data from the "European Randomized Study of Screening for Prostate Cancer" (ERSPC) suggests that PSA-based screening reduced the rate of death from prostate cancer by 20% [13]. Based on the results of these two large randomized trials, most of the major urological societies conclude that at present widespread mass screening for Pca is not appropriate [14]. According to the European Association of Urology Guidelines, early detection (opportunistic screening) should be offered to the well-informed men [14].

The American Cancer Society (ACS) recommends that men who are over 50 years of age and who are expected to live at least 10 more years should have a chance to make an informed decision with their health care provider about whether to be screened for Pca or not [15]. The decision should be made after getting information about the uncertainties, risks, and potential benefits of Pca screening. The ACS interestingly underlines that men should not be screened unless they have received this information [15]. According to the ACS, this discussion should take place starting at age 40 for men at high risk of developing Pca. This includes African Americans and men who have a first-degree relative (father, uncle, brother) diagnosed with Pca at an early age (younger than age 65) [15]. And finally, after this discussion, those men who want to be screened are suggested to be tested with the serum PSA as well as digital rectal exam (DRE) [15]. The ACS also suggests that men without symptoms of Pca who do not have a 10-year life expectancy should not be offered testing since they are not likely to benefit because it is generally considered that prostate cancer growth is slow [15]. However, a recently published report from three decades of followup of the natural history of Pca underlines that, although localized Pca most often has an indolent course, local progression and distant metastasis can develop over the long term, even among patients considered to be at low risk at diagnosis [16]. In this study, 38 (17%) of the 223 untreated men with localized Pca died because of prostate cancer after 32 years of followup [16]. The authors observed 90 (41.4%) local progression events and 41 (18.4%) cases of progression to distant metastasis, and these findings further complicate discussions around screening [16].

There are even slight disparities among guidelines declared by the same country, the USA. In the very recent annual meeting of the American Urological Association, guidelines on the early detection of Pca have been presented and some small changes are underlined [17]. According to this very recent declaration, in men aged 40–54 at average risk for the disease, the guidelines recommends that screening, as a routine practice, should not be encouraged. The Guidelines Committee underlines that evidence for the benefit for screening in this age range was limited while the quality and strength of the evidence regarding the harms of screening was high. In addition, routine screening were not recommended in men over the age of 70 or those with less than a 10-year life expectancy. However, the AUA guidelines acknowledged that some men over the age of 70 in excellent health might benefit from screening. In this setting, the guidelines suggest that a discussion of the unique risks and benefits of screening in older men occur. The same guidelines also point out that the highest quality evidence for benefit (defined as lower prostate cancer mortality) of screening was found in men aged 55 to 69 years. In men aged between 55 and 69 years, the guidelines strongly recommended shared decision making and screening based on a man's values and preferences. The only difference in the new guidelines is that they now recommend biennial screening to reduce the potential harms of screening. And interestingly the new AUA Guidelines stand out to be in disagreement with the US Preventive Services Task Force in recommendation against Pca screening in all men, regardless of age or risk, without even considering a discussion of the risks and benefits of screening. The U.S. Preventive Services Task Force recommends against PSA-based screening for Pca as a grade D recommendation and this recommendation applies to men in the general U.S. population, regardless of age [18]. However, the AUA continues to support a man's right to be tested for Pca and to have the insurance pay for it, if medically necessary [17].

Another debate going on around early detection is that we still do not clearly know the consequences of the treatment of early Pca detected by PSA screening. As mentioned above, it is evident that PSA-based screening results in reduction in prostate cancer-specific mortality, but it is associated with harm related to subsequent evaluation and treatments, some of which may be unnecessary [11, 18]. Therefore, informing a potential patient about the risks and benefits of screening is highly suggested and individual risk assessment is supported.

## 3. New Biomarkers

Research for a new marker has focused on serum-based, tissue-based and urine-based markers [19–21]. Despite extensive research efforts, very few biomarkers of Pca have been successfully implemented into clinical practice today and serum PSA test is still the most important biomarker for the detection and followup of Pca. Numerous studies of serum-, tissue, and urine-based prostate cancer biomarker candidates have been presented the last ten years [19–21]. It is generally accepted that unmet biomarker for prostate cancer should be addressed to distinguish BPH from Pca, to detect the aggressive forms from the indolent cases, and to identify the metastatic cancer predictors. However, biomarkers for identifying the most aggressive subsets of this malignancy are still missing. Briefly, PSA isoforms, pHi, and other combinations seem to be promising among serum-based biomarker. Tissue-based biomarkers are classified as diagnostic dyes, which are generally used to differentiate cancer with PIN and atypia, and prognostic biomarkers, which are usually determined on prostatic tissue using different techniques and are far from being a screening tool [20]. Out of urine-based marker, PCA-3 test is already in current daily practice and highly satisfactory results are being reported [21].

PCA-3 test has recently been approved by FDA [21, 22]. PCA-3 test is a urine-based marker, in which urine collected after a rectal exam and prostatic massage is highly specific for Pca, and is not affected by prostate volume and chronic prostatitis. It is also considered to be helpful in deciding rebiopsies and in the followup of patients under AS [22]. *PCA3* is a non coding RNA and the marker most specific to Pca that is clinically available to date. *PCA3* RNA expression is restricted to the prostate, and it is not expressed in any other normal human tissue or tumor. PCA3 RNA is highly overexpressed in 95% of tumors compared to normal or benign hyperplastic prostate tissue. Hessels et al. reported a median of 66-fold upregulation of *PCA3* in PCa tissue compared with normal prostate tissue [23].

To assess the probability of PCa detection on prostate biopsy, the quantitative PCA3 score was developed. The score is defined as the ratio (*PCA3* mRNA/PSA mRNA X1,000), meaning that *PCA3* expression is normalized with PSA expression serving as a prostate housekeeping gene [23]. Since a PCA3 score of 35 yielded the greatest diagnostic usefulness, demonstrating the optimal balance between specificity and sensitivity, it was adopted as a cut-off score. The average sensitivity and specificity of the PCA3 urine test is relatively high at 66% and 76%, respectively, versus 47% specificity for serum PSA [24].

To increase the predictive accuracy of the biopsy outcome and identify men at risk for PCa, novel biopsy nomograms were created, including that for *PCA3*. Auprich et al. have recently assessed the accuracy of the previously reported *PCA3*-based nomogram in a large European cohort of men [25]. The nomogram helped identify PCa in 255 of 621 men (41.1%) [25].

Another promising marker looks for an abnormal gene change called *TMPRSS2:ERG* in prostate cells [26, 27]. Gene alterations involving androgen regulated *TMPRSS2* and *ETS* transcription factor genes were identified in prostate cancer patients. *TMPRSS2* fusion with the *ETS* family member, an *ERG*, is the predominant variant in approximately 40% to 70% (about 50%) of patients with PCa. *ERG* is regarded as a key PCa oncogene. Considering the high prevalence of PCa, *TMPRSS2:ERG* fusion is the most common genetic aberration described to date in human solid tumors [27]. The cells to be tested are found in urine collected after a rectal exam. This gene change is found in more than 50% of all localized prostate cancers [26]. It is rarely found in the cells of men without prostate cancer. TMPRSS2-ERG has a specificity of 97% and sensitivity of 96%, and currently it is commercially available for clinical use in the US, and Europe [26].

In a very recent PubMed and Web of Science database search of the peer reviewed literature on urine-based testing for Pca, in an attempt toward the detection of Pca in urine, investigators have identified PCA3 and TMPRSS2:ERG fusion transcripts as promising RNA markers for cancer detection and possibly prognosis [28].

## 4. Genetics and Risk Assessment

In relation to investigations on genetic-based biomarkers, the key to curing Pca will ultimately come from an understanding of the genetic basis of this disease. Therefore, investigators have focused on identifying and isolating the gene, or genes, responsible for Pca [29]. Several high-penetrance genetic variants have been identified in many genetic linkage and genome-wide association studies around the world [29]. Many polymorphisms in genes, such as ELAC2 (locus HPC2), RNase L (locus hereditary prostate cancer 1 gene (HPC1s)), and MSR1, have been recognized as important genetic factors that confer an increased risk of developing Pca in many populations [29]. Tests to find abnormal Pca genes could also help identify men at high risk who would benefit from more intensive screening or from chemoprevention trials. Creation of a personalized panel of single-nucleotide polymorphisms (SNP) biomarkers may be important for the early and accurate detection of this cancer [30]. As a result, the need for a good biomarker is required to detect Pca earlier and to provide tools to follow patients during the early stages of the cancer. Furthermore, the use of a biomarker combination panel needs to be considered, in order to increase diagnostic accuracy.

A big enigma now facing men with prostate cancer and their doctors is figuring out which cancers are likely to stay within the gland and which are more likely to grow, spread, and definitely need treatment. In other words, worldwide accepted criteria to define low-intermediate-high risk prostate cancer are needed. The definition of high risk, which is still a matter of debate, was classically defined by Bastian et al. as any combination of the following factors: a prostate-specific antigen (PSA) score >20 ng/mL, a Gleason score of 8–10, or clinical stage T2C or greater [31]. Patients with high risk disease, which accounts for ≤15% of all new diagnoses, are more or less the focus of radical prostatectomy, either as mono- or multimodel therapy concept [31].

The potential complications of overdiagnosis and overtreatment have resulted in arguments about screening and introduced a new management approach called active surveillance as summarized above. The recent discovery of more than the 30 so-called prostate cancer susceptibility genes suggests the possibility of targeted screening of those men who have the highest risk of developing the aggressive form of Pca [28–30]. This could eventually help us to tell which men need treatment and which might be better served by active surveillance. For example, the product of a gene known as *EZH2* seems to appear more often in advanced prostate cancers than in those at an early stage [32]. Further studies will also be performed to try to block, or modify, the offending genes in order to prevent or alter the progression of prostate cancer.

## 5. Promising New Medical Treatment Options

On the other hand "Gene Therapy," which is a process of introducing genetically engineered material, usually DNA, into the body, is an evolving treatment option for Pca, but currently for advanced disease [33]. In a recently published report, experts reviewed the progress being made in gene therapy for Pca [33]. Overall, most of the more than 90 clinical protocols using gene therapy in Pca cancer patients in the National Institutes of Health database used adenoviral vectors [33]. While adenoviral gene therapy strategies in Pca patients were proved to be safe thus far, scientists are still struggling to identify which approaches should be considered and improved. However, experts must first conduct randomized, well-controlled Phase 3 trials, and that point has not yet been reached [33, 34].

Virus therapy, also known as "oncolytic virus therapy," is a new potential treatment strategy for advanced prostate cancer patients and is still in the early stages of investigation. A virus chosen to treat cancer is called an oncolytic virus, and once it is introduced to the prostate cancer cells, it replicates and kills tumor cells selectively [35]. The progeny viruses produced within the cancer cells are then released, and they spread and infect other cancer cells that surround it. This cycle continues and results in the killing of more and more cancer cells. Because oncolytic viruses are not able to replicate in healthy cells, normal tissue is not damaged [35, 36]. Experts believe that the development of oncolytic virus therapy will eventually lead to a promising treatment option for men who have Pca, but ethical issues prevent these investigations among men with localized Pca.

There are also two "vaccines" commercially available for the management of advanced stage Pca [37, 38]. However, vaccines to prevent the disease in the early stage are awaited. Unlike vaccines against infections like measles or mumps, these currently available vaccines are designed to help treat, not prevent, prostate cancer. An example of this type of vaccine is Sipuleucel-T (Provenge), which has received FDA approval. Although clinical experience with this vaccine is limited, it has been shown to improve survival in patients whose cancer has become resistant to hormones. However, the cost of each treatment course is enormous—about $100,000, because doses of Sipuleucel-T are unique and individually prepared for each patient.

The other available Pca vaccine (PROSTVAC-VF) uses a virus that has been genetically modified to contain PSA but is still investigational. The patient's immune system should respond to the virus and begin to recognize and destroy cancer cells containing PSA. Early results with this vaccine have been promising [38]. Several other prostate cancer vaccines are also in development.

There are great advances in the medical treatment of advanced and metastatic disease, but this topic is out of the scope of this review. However, once the efficacy of these new compounds for advanced and incurable disease has been established, these agents may be explored as an adjuvant and neoadjuvant treatment in order to increase the chance of cure for localized disease. And abiraterone, especially, a new compound used for metastatic disease may be offered to patients with localized prostate cancer who refuse radical treatment options.

## 6. Life Style and Diet

Life style and dietary alterations are also believed to alter the progression of prostate cancer [39]. Observational evidence show that there is a relationship between the so-called energy balance factors (i.e., diet, physical activity, and body weight) and risk of cancer recurrence as well as mortality in cancers of the breast, prostate, colon, and, perhaps, other cancers. Furthermore, individuals who make favorable changes in these lifestyle factors after cancer diagnosis feel better, experience less fatigue, and may possibly even decrease risk of cancer recurrence [39]. Other lifestyle behaviors, such as smoking and alcohol consumption, have also been linked to the development of common cancers and may have important health consequences for cancer survivors. An interesting study has shown that in men with a rising PSA level after surgery or radiation therapy, drinking pomegranate juice seemed to slow the time it took the PSA level to double [40]. Larger studies are now trying to confirm these results. Supporting the role of pomegranate as a strong antioxidant, investigators highly suggest the use of pomegranate extracts in the therapy of erectile dysfunction, benign prostatic hyperplasia, and Pca [41]. Therefore, patients with localized Pca may also be advised to consume pomegranate juice.

Some encouraging early results have also been reported with flaxseed supplements. Studies indicate that enterolactone and enterodiol, mammalian lignans derived from dietary sources such as flaxseed, sesame seeds, kale, broccoli, and apricots, may impede tumor proliferation by inhibiting activation of nuclear factor kappa B (NF$\kappa$B) and vascular endothelial growth factor (VEGF) [42]. One randomized controlled study in men with early Pca before surgery found that daily flaxseed seemed to slow the rate at which Pca cells multiplied [43]. More research is needed to confirm this finding. Another study found that men who chose not to have treatment for their localized Pca may be able to slow its growth with intensive lifestyle changes [44].

The men ate a vegan diet (no meat, fish, eggs, or dairy products) and exercised frequently, and the authors observed a slight diminishment in the serum PSA levels after one year. However, it is not known if this effect will last longer since the report only followed the men for 1 year.

## 7. Advances in Diagnosis

Researchers also keep on searching how to improve the diagnostic accuracy of transrectal ultrasound guided biopsy (TRUS-bx), which currently the basic way to diagnose Pca [45]. It is well-known that standard ultrasound may not detect some areas containing cancer. Therefore, a newer approach is to measure blood flow within the gland using a technique called "*color Doppler ultrasound*" since tumors often have more blood vessels around them than normal tissue. It may make prostate biopsies more accurate by helping to ensure that the right part of the gland is sampled. An even newer technique may enhance color Doppler further, called "contrast enhanced color Doppler US." It involves first injecting the patient with a contrast agent containing microbubbles. Promising results have been reported, but more studies are needed before its use becomes common [45].

Apart from a possible role in the diagnosis of PCa, elasticity imaging techniques may monitor high intensity focused ultrasound (HIFU) results in prostate cancer, because HIFU-ablated lesions are stiffer than the surrounding normal untreated tissue [45]. Promising results have recently been published, but further clinical trials are needed before this application can be considered established.

There are increasing number of publications regarding the use of MRI in the diagnosis of prostate cancer [46, 47]. Magnetic resonance (MR) imaging currently plays a pivotal role in pretreatment assessment of prostate cancer. Multiparametric MR imaging, a combination of anatomic and functional MR imaging techniques (diffusion-weighted imaging, dynamic contrast material-enhanced imaging, and MR spectroscopy), significantly improves the accuracy of tumor localization and local staging [48]. MRI anatomic imaging with spectroscopic evaluation analyzes cellular metabolites within the prostate and their changes in PCa [49]. In the prostate, choline and citrate are the important metabolites [49]. Choline is an important component of cell membranes, integrated into the phospholipid bilayer. Prostate malignancy is hypothesized to lead to increased choline because of increased cell proliferation. Citrate is a component of the citric acid cycle that normally accumulates within the glandular ducts formed by prostate epithelial cells. Prostate malignancy is thought to lead to decreased choline levels by means of increased tumor metabolic activity and decreased glandular differentiation [49]. An accuracy up to 90% has been reported with dynamic contrast-enhanced MRI in detection and localization of prostate [48]. Therefore, MRI can especially help to guide prostate biopsies in men who previously had negative TRUS-guided biopsies [47, 49, 50]. In a very recent paper, the role of "MRI-targeted TRUS-guided transperineal fusion biopsy" in the diagnosis of Pca was evaluated in 347 consecutive patients [50]. The majority of these patients had a history of negative TRUS-guided biopsies. In the study, all patients underwent multiparametric (mp) MRI at 3T and received systematic stereotactic prostate biopsies plus MRI-targeted TRUS-guided biopsies in case of MRI abnormalities [50]. The investigators were able identify Pca in 58% of the samples and concluded that MRI-targeted TRUS-guided transperineal fusion biopsy provides high detection rates of clinically significant tumors. However, they also underline that this technique still has some limitations, and therefore systematic biopsies should currently not be omitted [50]. Similarly, a median Pca detection rate of 42% has been reported in a recent meta-analysis [51].

Another advantage offered by new MRI technologies is that anatomic MR imaging provides highly accurate local staging information, particularly about extraprostatic extension and seminal vesicle invasion for pretreatment planning (especially for external beam radiotherapy and brachytherapy) [48]. The dominant intraprostatic tumor and local recurrence in the prostatectomy bed can be better localized with multiparametric MR imaging for dose painting [48]. MRI can also be used in early posttreatment evaluation after brachytherapy [48].

Furthermore, MRI is becoming more important in the followup of patients under AS [52]. *Enhanced MRI* may also help us to detect lymph nodes that contain cancer better than conventional CT and MRI. A newer type of positron-emission tomography PET scan that uses radioactive carbon acetate instead of FDG may also be helpful in detecting Pca in different parts of the body, as well as helping to determine if treatment has been effective [49]. Studies of this technique are now in progress [49].

## 8. Active Surveillance

In addition to advances in the screening, prevention, and diagnosis of Pca, researchers spent a big effort on treatment options and their comparative results. Despite a large number of publications on this area, little is known about the relative effectiveness and harms of treatments because of the paucity of randomized controlled trials. Recently, the Departments of Veterans Affairs/National Cancer Institute/Agency for Healthcare Research and Quality Cooperative Studies Program Study no. 407: Prostate Cancer Intervention Versus Observation Trial (PIVOT) reported a multicenter randomized controlled trial, initiated in 1994, comparing *radical prostatectomy* with *watchful waiting* in men with clinically localized Pca [11]. In this large study, a total of 13.022 men with prostate cancer at 52 US medical centers were considered for potential enrollment and a total of 731 men agreed to participate and were randomized [11]. PIVOT enrolled an ethnically diverse population representative of men diagnosed with Pca in the United States. During the median followup of 10.0 years, 171 of 364 men (47.0%) assigned to radical prostatectomy died, as compared with 183 of 367 (49.9%) assigned to observation. Among men assigned to radical prostatectomy, 21 (5.8%) died from prostate cancer or treatment, as compared with 31 men (8.4%) assigned to observation. The effect of treatment on all-cause and Pca

mortality did not differ according to age, race, coexisting conditions, self-reported performance status, or histologic features of the tumor. Radical prostatectomy was associated with reduced all-cause mortality among men with a PSA value greater than 10 ng per milliliter and possibly among those with intermediate-risk or high-risk tumors. As a conclusion, the authors state that radical prostatectomy did not significantly reduce all-cause or Pca mortality, as compared with observation, among men with localized prostate cancer detected during the early era of PSA testing [11].

However, in a previous paper from the Scandinavian prostate cancer group, comparing radical prostatectomy and watchful waiting, it was concluded that radical prostatectomy reduces Pca mortality and risk of metastases with little or no further increase in benefit 10 or more years after surgery [53]. Comparison of the data sample of eligible men declining PIVOT participation as well as to men enrolled in the Scandinavian trial indicated that PIVOT enrollees are representative of men being diagnosed and treated in the United States and are quite different from men in the Scandinavian trial [11, 53].

Basically taking the results of the PIVOT study and the Scandinavian study as well as the natural history of prostate cancer into consideration, a relatively new management concept called "active surveillance" has been introduced into the practice. In this new management concept, definitive treatment options of localized prostate cancer are deferred until certain level of progression with the patient under close control with serial serum PSA analyzes and repeats TRUS-guided prostate biopsies [3]. Recent findings suggest that detailed MRI studies as well as new prostate cancer markers such as PCA-3 test are helpful in the followup of patients under AS and especially in defining progression which is an absolute indication for the timing definitive treatment [47, 49].

AS means deferring treatment initially for a growing proportion of men diagnosed with low-risk (i.e., low volume, stage, and grade) Pca [54]. However, there is no worldwide accepted consensus on defining exact criteria in order to offer active surveillance to men with Pca [55]. Different institutions use different criteria to include men into active surveillance protocol [55]. In general, patients with PSA < 10, Gleason score <3 + 3 or 3 + 4, and less than 3 positive cores on TRUS biopsy are candidates for active surveillance. Men under active surveillance are followed carefully with serial PSA assessments, repeated biopsies, and in some cases other tests intended to identify early signs of progression (such as MRI and biomarkers).

The term "active surveillance" has supplanted "watchful waiting," but the two are not synonymous. The latter term is generally applied to older men with significant comorbidity, who were advised to defer treatment unless symptoms of advanced disease developed, at which point palliative androgen deprivation could be offered. Active surveillance, on the other hand, rests on the presumptions that the lead time from diagnosis to clinical progression is usually long for low risk disease and that at the first signs of higher-risk disease the cancer can be treated, within the window of opportunity for cure [54, 55].

## 9. Radical Prostatectomy

Although new developments are being waited to be introduced into practice about biomarkers and genetics, the surgical treatment (radical prostatectomy), which is currently considered to be the gold standard in the management of localized Pca, has almost achieved its excellence since its first anatomical description by Walsh more than 30 years ago [3, 56]. The overall 25-year progression-free, metastasis-free, and cancer-specific survival rates after anatomical radical prostatectomy were 68%, 84%, and 86%, respectively, although there were significant differences in treatment outcomes between men treated in the pre-PSA and PSA eras. In each era, there were significant differences in progression-free, metastasis-free, and cancer-specific survival [56]. Therefore, the authors conclude that anatomical radical retropubic prostatectomy continues to represent the gold standard in the surgical management of clinically localized Pca to which alternate treatment options should be compared.

Minimally invasive laparoscopic surgery and especially "robot-assisted laparoscopic radical prostatectomy" has also contributed a lot to the management of localized Pca. Robot-assisted radical prostatectomy (RARP) is gaining increasing acceptance among urologists and especially among patients because of widespread advertisements, and it has become the dominant technique in the United States despite a paucity of prospective studies or randomized trials supporting its superiority over RRP [57]. Although there is no prospectively randomization with open radical prostatectomy, experts indicate that there is sufficient evidence in order to suggest RARP as a valuable therapeutic option for clinically localized PCa [57].

Further developments in robot assisted surgery such as single port surgery may add some advantages to the surgical management of localized Pca, but these techniques have some serious limitations such as a very long learning curve and lack of ideal instruments. With all these high technological advances, expectations of patients with localized Pca have also increased accordingly. These high expectations were recently summarized and analyzed as "Trifecta" and more recently as "Pentafecta" [58, 59]. Authors briefly believe that "Pentafecta (cancer control, continence, and potency, no postoperative complications, negative surgical margins)" outcomes accurately represent patients' expectations after minimally invasive surgery for Pca and that this definition is highly beneficial and can be used when counseling patients with clinically localized disease [59]. And more recently, an outstanding group of authors with high expertise in this field have introduced the survival, continence, and potency (SCP) classification in order to report the oncologic and functional outcomes [60].

## 10. Radiation Therapy

Another radical treatment option for Pca is radiation therapy [3]. Advances in technology are making it possible to target radiation for Pca more precisely than in the past [61]. Currently used methods such as conformal radiation therapy (CRT), intensity modulated radiation therapy (IMRT),

and proton beam radiation allow to treat only the prostate gland and avoid radiation to normal surrounding tissues as much as possible. These methods are expected to increase the effectiveness of radiation therapy while reducing the side effects. Studies are being done to find out which radiation techniques are best suited for specific groups of patients with Pca. There are also many studies under process in order to improve the effectiveness of radiation therapy. So far, though, no study has arised. Recently, a linear accelerator (CT-linac) has been introduced to improve results of radiotherapy especially when prostate movements are problematic for intensity-modulated radiotherapy [62].

## 11. New Horizons: Focal Therapy

Another area of research is "focal therapy" for localized Pca. This approach attempts to mirror the evolution of breast cancer treatment, which often involves "lumpectomy" as part of the initial management of the disease. Similarly, "partial nephrectomy" for small renal masses also represent a logical model for focal therapy in localized Pca. Focal therapy involves treatment of only that part of the prostate that is affected by cancer and uses methods like cryotherapy, high intensity focused ultrasonography (HIFU), and brachytherapy (seed implantation) to treat the cancer [63]. Several energy modalities are being developed to achieve the trifecta of continence, potency, and oncologic efficiency [63]. Focal therapy is still at its infancy and its role is unclear because of unresolved problems related to the lack of a proper method for complete evaluation of cancer location within the prostate and the potential coexistence of many different cancerous areas within the same prostate. These alternatives are still considered to be "experimental" in guidelines [3]. However, with the advances in imaging and especially in MRI, this approach will find a special place between surveillance and radical therapies in the management of localized Pca. In a recent review, it was underlined that guidance of thermal therapies for focal ablation of Pca will likely prove critically dependent on MRI functioning in four separate roles, summarized as device positioning, thermal monitoring of prostate ablation, and depiction of ablated prostate tissue [64]. A fourth critical role, identification of cancer within the gland for targeting of thermal therapy, is more problematic at present but will likely become practical with further technological advances [64].

As a conclusion, the management of localized Pca has dramatically changed in the last decades. However, further revolutionary changes in the diagnosis and management of Pca are awaited in the near future. It may be difficult to define a worldwide accepted screening policy because of different health systems in each country but new markers will soon be available in the market in order to increase the specificity and sensitivity in the diagnosis of Pca. Investigators have focused on identifying and isolating the gene, or genes, responsible for prostate cancer, and this will obviously help us to understand the basics of Pca. There are several promising medical treatment options, which are already used or under investigation for the management of metastatic Pca. But researchers postulate that these new alternatives may get involved in the management of localized Pca in the future. In addition to investigations in order to prevent Pca, it is also clear that life style and diet modifications will help us to decrease the prevalence of Pca. Advances in diagnostic techniques will probably help us to define the disease in the earlier stage in a less morbid way and will probably let us decide whether to do active surveillance or perform treatment especially with minimal invasive focal treatment options in the majority of cases.

## References


[1] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics, 2012," *CA: A Cancer Journal for Clinicians*, vol. 62, no. 1, pp. 10–29, 2012.

[2] A. B. Jani, P. A. S. Johnstone, S. L. Liauw, V. A. Master, and O. W. Brawley, "Age and grade trends in prostate cancer (1974–2003): a surveillance, epidemiology, and end results registry analysis," *American Journal of Clinical Oncology*, vol. 31, no. 4, pp. 375–378, 2008.

[3] A. Heidenreich, J. Bellmunt, M. Bolla et al., "EAU guidelines on prostate cancer—part 1: screening, diagnosis, and treatment of clinically localised disease," *European Urology*, vol. 59, no. 1, pp. 61–71, 2011.

[4] J. Xia, R. Gulati, M. Au, J. L. Gore, D. W. Lin, and R. Etzioni, "Effects of screening on radical prostatectomy efficacy: the prostate cancer intervention versus observation trial," *The Journal of the National Cancer Institute*, vol. 105, no. 8, pp. 546–550, 2013.

[5] E. Killick, E. Bancroft, Z. Kote-Jarai, and R. Eeles, "Beyond prostate-specific antigen—future biomarkers for the early detection and management of prostate cancer," *Clinical Oncology*, vol. 24, no. 8, pp. 545–555, 2012.

[6] W. J. Catalona, A. W. Partin, M. G. Sanda et al., "A multicenter study of [-2]pro-prostate specific antigen combined with prostate specific antigen and free prostate specific antigen for prostate cancer detection in the 2.0 to 10.0 ng/ml prostate specific antigen range," *Journal of Urology*, vol. 185, no. 5, pp. 1650–1655, 2011.

[7] W. J. Catalona, G. Bartsch, H. G. Rittenhouse et al., "Serum pro-prostate specific antigen preferentially detects aggressive prostate cancers in men with 2 to 4 ng/ml prostate specific antigen," *Journal of Urology*, vol. 171, no. 6, part 1, pp. 2239–2244, 2004.

[8] C. Stephan, A.-M. Kahrs, H. Cammann et al., "A [-2] pro PSA-based artificial neural network significantly improves differentiation between prostate cancer and benign prostatic diseases," *Prostate*, vol. 69, no. 2, pp. 198–207, 2009.

[9] D. V. Makarov, S. Isharwal, L. J. Sokoll et al., "Pro-prostate-specific antigen measurements in serum and tissue are associated with treatment necessity among men enrolled in expectant management for prostate cancer," *Clinical Cancer Research*, vol. 15, no. 23, pp. 7316–7321, 2009.

[10] S. F. Shariat, A. Semjonow, H. Lilja, C. Savage, A. J. Vickers, and A. Bjartell, "Tumor markers in prostate cancer I: blood-based markers," *Acta Oncologica*, vol. 50, supplement 1, pp. 61–75, 2011.

[11] T. J. Wilt, M. K. Brawer, K. M. Jones et al., "Radical prostatectomy versus observation for localized prostate cancer," *The New England Journal of Medicine*, vol. 367, no. 3, pp. 203–213, 2012.

[12] G. L. Andriole, E. D. Crawford, R. L. Grub et al., "Mortality results from a randomized prostate cancer screening trial,"

*The New England Journal of Medicine*, vol. 360, no. 13, pp. 1310–1319, 2009.

[13] F. H. Schröder, J. Hugosson, M. J. Roobol et al., "Screening and prostate-cancer mortality in a randomized european study," *The New England Journal of Medicine*, vol. 360, no. 13, pp. 1320–1328, 2009.

[14] A. Heidenreich, P. A. Abrahamsson, W. Artibani et al., "Early detection of prostate cancer: European association of urology recommendation," *European Urology*, vol. 64, no. 3, pp. 347–354, 2013.

[15] A. M. Wolf, R. C. Wender, R. B. Etzioni et al., "American Cancer Society guideline for the early detection of prostate cancer: update 2010," *CA: A Cancer Journal for Clinicians*, vol. 60, no. 2, pp. 70–98, 2010.

[16] M. Popiolek, J. R. Rider, O. Andrén et al., "Natural history of early, localized prostate cancer: a final report from three decades of follow-up," *European Urology*, vol. 63, no. 3, pp. 428–435, 2013.

[17] H. B. Carter, P. C. Albertsen, M. J. Barry et al., "Early detection of prostate cancer: AUA guideline," *The Journal of Urology*, vol. 190, no. 2, pp. 419–426, 2013.

[18] V. A. Moyer, "U.S. Preventive Services Task Force. Screening for prostate cancer: U.S. Preventive Services Task Force recommendation statement," *Annals of Internal Medicine*, vol. 157, no. 2, pp. 120–134, 2012.

[19] W. Artibani, "Landmarks in prostate cancer diagnosis: the biomarkers," *BJU International*, vol. 110, supplement 1, pp. 8–13, 2012.

[20] A. Bjartell, R. Montironi, D. M. Berney, and L. Egevad, "Tumour markers in prostate cancer II: diagnostic and prognostic cellular biomarkers," *Acta Oncologica*, vol. 50, supplement 1, pp. 76–84, 2011.

[21] M. J. Roobol, A. Haese, and A. Bjartell, "Tumour markers in prostate cancer III: biomarkers in urine," *Acta Oncologica*, vol. 50, supplement 1, pp. 85–89, 2011.

[22] M. Auprich, A. Bjartell, F. K.-H. Chun et al., "Contemporary role of prostate cancer antigen 3 in the management of prostate cancer," *European Urology*, vol. 60, no. 5, pp. 1045–1054, 2011.

[23] D. Hessels, J. M. Klein Gunnewiek, I. van Oort et al., "DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer," *European Urology*, vol. 44, no. 1, pp. 8–15, 2003.

[24] M. P. M. Q. van Gils, D. Hessels, O. van Hooij et al., "The time-resolved fluorescence-based PCA3 test on urinary sediments after digital rectal examination; a Dutch multicenter validation of the diagnostic performance," *Clinical Cancer Research*, vol. 13, no. 3, pp. 939–943, 2007.

[25] M. Auprich, A. Haese, J. Walz et al., "External validation of urinary PCA3-based nomograms to individually predict prostate biopsy outcome," *European Urology*, vol. 58, no. 5, pp. 727–732, 2010.

[26] M. Salagierski and J. A. Schalken, "Molecular diagnosis of prostate cancer: PCA3 and TMPRSS2:ERG gene fusion," *Journal of Urology*, vol. 187, no. 3, pp. 795–801, 2012.

[27] S. Perner, J.-M. Mosquera, F. Demichelis et al., "TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion," *American Journal of Surgical Pathology*, vol. 31, no. 6, pp. 882–888, 2007.

[28] M. J. Alvarez-Cubero, M. Saiz, L. J. Martinez-Gonzalez, J. C. Alvarez, J. A. Lorente, and J. M. Cozar, "Genetic analysis of the principal genes related to prostate cancer: a review," *Urologic Oncology*, 2012.

[29] M. J. Alvarez-Cubero, M. Saiz, L. J. Martinez-Gonzalez, J. C. Alvarez, J. A. Lorente, and J. M. Cozar, "Genetic analysis of the principal genes related to prostate cancer: a review," *Urologic Oncology*, 2012.

[30] F. Schmalfuss and P. L. Kolominsky-Rabas, "Personalized medicine in screening for malignant disease: a review of methods and applications," *Biomark Insights*, vol. 8, pp. 9–14, 2013.

[31] P. J. Bastian, S. A. Boorjian, A. Bossi et al., "High-risk prostate cancer: from definition to contemporary management," *European Urology*, vol. 61, no. 6, pp. 1096–1106, 2012.

[32] S. Dijkstra, A. R. Hamid, G. H. Leyten, and J. A. Schalken, "Personalized management in low-risk prostate cancer: the role of biomarkers," *Prostate Cancer*, vol. 2012, Article ID 327104, 7 pages, 2012.

[33] B. D. W. Karanikolas, M. L. Figueiredo, and L. Wu, "Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines," *Prostate*, vol. 70, no. 6, pp. 675–688, 2010.

[34] S. Kochanek and B. Gansbacher, "Prostate cancer gene therapy: attempts to innovate," *Human Gene Therapy*, vol. 21, no. 7, p. 791, 2010.

[35] K. A. Ahmed, B. J. Davis, T. M. Wilson, G. A. Wiseman, M. J. Federspiel, and J. C. Morris, "Progress in gene therapy for prostate cancer," *Frontiers in Oncology*, vol. 2, article 172, 2012.

[36] H. Fukuhara, Y. Homma, and T. Todo, "Oncolytic virus therapy for prostate cancer: review article," *International Journal of Urology*, vol. 17, no. 1, pp. 20–30, 2010.

[37] P. Msaouel, I. D. Iankov, C. Allen et al., "Engineered measles virus as a novel oncolytic therapy against prostate cancer," *Prostate*, vol. 69, no. 1, pp. 82–91, 2009.

[38] P. Kawalec, A. Paszulewicz, P. Holko, and A. Pilc, "Sipuleucel-T immunotherapy for castration-resistant prostate cancer. A systematic review and meta-analysis," *Archives of Medical Science*, vol. 8, no. 5, pp. 767–775, 2012.

[39] R. A. Madan, P. M. Arlen, M. Mohebtash, J. W. Hodge, and J. L. Gulley, "Prostvac-VF: a vector-based vaccine targeting PSA in prostate cancer," *Expert Opinion on Investigational Drugs*, vol. 18, no. 7, pp. 1001–1011, 2009.

[40] J. Ligibel, "Lifestyle factors in cancer survivorship," *Journal of Clinical Oncology*, vol. 30, no. 30, pp. 3697–3704, 2012.

[41] C. J. Paller, X. Ye, P. J. Wozniak et al., "A randomized phase II study of pomegranate extract for men with rising PSA following initial therapy for localizedprostate cancer," *Prostate Cancer and Prostatic Diseases*, vol. 16, no. 1, pp. 50–55, 2013.

[42] N. Kroeger, A. S. Belldegrun, and A. J. Pantuck, "Pomegranate extracts in the management of men's urologic health: scientific rationale and preclinical and clinical data," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 701434, 9 pages, 2013.

[43] M. Azrad, R. T. Vollmer, J. Madden et al., "Flaxseed-derived enterolactone is inversely associated with tumor cell proliferation in men with localized prostate cancer," *Journal of Medicinal Food*, vol. 16, no. 4, pp. 357–360, 2013.

[44] W. Demark-Wahnefried, T. J. Polascik, S. L. George et al., "Flaxseed supplementation (not dietary fat restriction) reduces prostate cancer proliferation rates in men presurgery," *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 12, pp. 3577–3587, 2008.

[45] S. E. Berkow, N. D. Barnard, G. A. Saxe, and T. Ankerberg-Nobis, "Diet and survival after prostate cancer diagnosis," *Nutrition Reviews*, vol. 65, no. 9, pp. 391–403, 2007.

[46] S. Gravas, C. Mamoulakis, J. Rioja et al., "Advances in ultrasound technology in oncologic urology," *Urologic Clinics of North America*, vol. 36, no. 2, pp. 133–145, 2009.

[47] P. Boonsirikamchai, S. Choi, S. J. Frank et al., "MR imaging of prostate cancer in radiation oncology: what radiologists need to know," *Radiographics*, vol. 33, no. 3, pp. 741–761, 2013.

[48] H. U. Ahmed, "Multiparametric magnetic resonance imaging findings in men with low-risk prostate cancer followed using active surveillance," *BJU International*, vol. 111, no. 7, pp. 1011–1013, 2013.

[49] L. Dickinson, H. U. Ahmed, and C. Allen, "Clinical applications of multiparametric MRI within the prostate cancer diagnostic pathway," *Urologic Oncology*, vol. 31, no. 3, pp. 281–284, 2013.

[50] M. S. Gee, M. G. Harisinghani, and S. Tabatabaei, "Molecular imaging in urologic surgery," *Urologic Clinics of North America*, vol. 36, no. 2, pp. 125–132, 2009.

[51] T. H. Kuru, M. C. Roethke, J. Seidenader et al., "Critical evaluation of MRI-targeted TRUS-guided transperineal fusion biopsy for detection of prostate cancer," *The Journal of Urology*, 2013.

[52] M. S. Gee, M. G. Harisinghani, and S. Tabatabaei, "Molecular imaging in urologic surgery," *Urologic Clinics of North America*, vol. 36, no. 2, pp. 125–132, 2009.

[53] C. G. Overduin, J. J. Fütterer, and J. O. Barentsz, "MRI-guided biopsy for prostate cancer detection: a systematic review of current clinical results," *Current Urology Reports*, vol. 14, no. 3, pp. 209–213, 2013.

[54] A. Bill-Axelson, L. Holmberg, F. Filén et al., "Radical prostatectomy versus watchful waiting in localized prostate cancer: the Scandinavian prostate cancer group-4 randomized trial," *Journal of the National Cancer Institute*, vol. 100, no. 16, pp. 1144–1154, 2008.

[55] L. Klotz, "Active surveillance for low-risk prostate cancer," *Medicine Reports*, vol. 4, article 16, 2012.

[56] M. A. Dall'Era, P. C. Albertsen, C. Bangma et al., "Active surveillance for prostate cancer: a systematic review of the literature," *European Urology*, vol. 62, pp. 976–983, 2012.

[57] J. K. Mullins, Z. Feng, B. J. Trock, J. I. Epstein, P. C. Walsh, and S. Loeb, "The impact of anatomical radical retropubic prostatectomy on cancer control: the 30-year anniversary," *Journal of Urology*, vol. 188, no. 6, pp. 2219–2224, 2012.

[58] F. Montorsi, T. G. Wilson, R. C. Rosen et al., "Best practices in robot-assisted radical prostatectomy: recommendations of the Pasadena Consensus Panel," *European Urology*, vol. 62, no. 3, pp. 368–381, 2012.

[59] F. J. Bianco Jr., P. T. Scardino, and J. A. Eastham, "Radical prostatectomy: long-term cancer control and recovery of sexual and urinary function ("trifecta")," *Urology*, vol. 66, supplement 5, pp. 83–94, 2005.

[60] V. R. Patel, A. Sivaraman, R. F. Coelho et al., "Pentafecta: a new concept for reporting outcomes of robot-assisted laparoscopic radical prostatectomy," *European Urology*, vol. 59, no. 5, pp. 702–707, 2011.

[61] V. Ficarra, P. Sooriakumaran, G. Novara et al., "Systematic review of methods for reporting combined outcomes after radical prostatectomy and proposal of a novel system: the survival, continence, and potency (SCP) classification," *European Urology*, vol. 61, no. 3, pp. 541–548, 2012.

[62] G. C. Morton, D. A. Loblaw, R. Sankreacha et al., "Single-fraction high-dose-rate brachytherapy and hypofractionated external beam radiotherapy for men with intermediate-risk prostate cancer: analysis of short- and medium-term toxicity and quality of life," *International Journal of Radiation Oncology Biology Physics*, vol. 77, no. 3, pp. 811–817, 2010.

[63] H. Aoyama, Y. Azuma, and K. Inamura, "Comparison of daily prostate positions during conformal radiation therapy of prostate cancer using an integrated CT-linear accelerator system: in-room CT image versus digitally reconstructed radiograph," *Journal of Radiation Research*, vol. 52, no. 2, pp. 220–228, 2011.

[64] G. Bozzini, P. Colin, P. Nevoux, A. Villers, S. Mordon, and N. Betrouni, "Focal therapy of prostate cancer: energies and procedures," *Urologic Oncology*, vol. 31, no. 2, pp. 155–167, 2013.

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*Research Article*

# Dual Silencing of Hsp27 and c-FLIP Enhances Doxazosin-Induced Apoptosis in PC-3 Prostate Cancer Cells

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We evaluated effect of dual gene silencing of Hsp27 and c-FLIP in doxazosin-induced apoptosis of PC-3 cell. After transfection using Hsp27 and c-FLIP siRNA mixture (dual silencing), doxazosin treatment was done at the concentrations of 1, 10, and 25 $\mu$M. We checked apoptosis of PC-3 cells with and TUNEL staining. We also checked interaction between Hsp27 and C-FLIP in the process of apoptosis inhibition. Spontaneous apoptotic index was 5% under single gene silencing of Hsp27 and c-FLIP and 7% under dual silencing of Hsp27 and c-FLIP. When doxazosin treatment was added, apoptotic indices increased in a dose-dependent manner (1, 10, and 25 $\mu$M): nonsilencing 10, 27, and 52%; Hsp27-silencing: 14, 35, and 68%; c-FLIP silencing: 21, 46, and 78%; dual silencing: 38, 76, and 92%. While c-FLIP gene expression decreased in Hsp27- silenced cells, Hsp27 gene expression showed markedly decreased pattern in the cells of c-FLIP silencing. The knockout of c-FLIP and Hsp27 genes together enhances apoptosis even under 1 $\mu$M, rather than low concentration, of doxazosin in PC-3 cells. This finding suggests a new strategy of multiple knockout of antiapoptotic and survival factors in the treatment of late-stage prostate cancer refractory to conventional therapy.

## 1. Introduction

Advanced prostate cancers eventually progress to the terminal stage of castration refractory prostate cancer (CRPC) which is not responsive to most of treatment modalities. Docetaxel-based chemotherapy has been the mainstay of treatment for this metastatic CRPC, and recently, other anticancer drugs such as cabazitaxel or abiraterone acetate are allowed for use, but their therapeutic benefits are still not that sufficient [1–4].

Researchers consider some defects in apoptotic signaling pathway or abnormal overexpression of antiapoptotic factors as the main causes of treatment resistance in patients with late-stage prostate cancer [5].

Among those antiapoptotic factors, bcl-2 [6], clusterin [7], heat shock prostein 27 (Hsp27) [8, 9], cellular-FLICE inhibitory protein (c-FLIP) [10], and GRP78 [11] have been widely reported. Overexpression of these factors could be induced by androgen deprivation therapy (ADT), chemotherapy, or other extreme stresses.

Recently, new therapeutic ways of blocking these factors are being developed as new drugs, and among them, clusterin ASO is now on Phase 3 clinical trial [12]. While knockdown of each factor alone can exert apoptosis inducing effect, blocking several factors which have some different pathways together may enhance apoptosis in prostate cancer cells. This concept can be applied to the development of new therapy against prostate cancer.

Hsp27, one of small Hsps, inhibits key effectors of the apoptotic pathway at the pre- and postmitochondria levels [13]. In prostate cancer, Hsp27 is associated with pathologic stage, Gleason score, lymph node metastasis, shorter biochemical recurrence, and poor clinical outcome [14, 15].

The c-FLIP is an inhibitor of apoptosis downstream of the death receptors Fas, DE4, and DR5 [16]. The expression of c-FLIP is closely related to the resistance to tumor necrosis

factor-related apoptosis-inducing ligand (TRAIL) and FAS-mediated apoptosis in prostate and bladder cancers [17–19]. Therefore, c-FLIP is regarded as a new therapeutic target for relevant cancers [20].

Dual silencing is reported to be effective in augmenting biologic effect on laboratory level and technically feasible [21].

Doxazosin, an quinazoline derivative $\alpha$1 adrenoreceptor antagonist, has been known to exert antitumor effect via induction of apoptosis in PC-3 cancer cells [22]. Doxazosin induces apoptosis via not an $\alpha$1-adrenoceptor-dependent action but a death receptor-mediated pathway [23].

In this present study, we investigated the enhanced effect of double knockout of Hsp27 and c-FLIP genes using siRNA technology in PC-3 prostate cancer cells and also tried to find out whether there is any interactive role between the 2 factors by observing the expression of one factor under silencing of the other factor.

## 2. Materials and Methods

*2.1. Cell Lines.* PC-3 cells obtained from American Type Culture Collection (Bethesda, MD, USA) were maintained in F-12 medium. We compared as group treated scrambled siRNA, AI-LNCaP-scr-siRNA.

*2.2. Doxazosin Treatment.* Doxazosin (Sigma Aldrich Korea, Seoul, Korea) was prepared as described in a previous study [7]. Cultures at 80% confluence were changed to fresh media and treated with doxazosin or serum-free media containing 0.25% DMSO as control.

*2.3. Transfection with siRNA.* The mRNA target sequences to Hsp27 (GeneBank Accession no. X54079.1) and c-FLIP (Gene ID: 8837) were designed using a siRNA template design tool (Ambion, Austin, TX, USA), and siRNA was prepared with a Silencer siRNA construction kit (Ambion). Three oligonucleotides Hsp27-1 (5′-GACCUACCGAGGAGCUUUCdTT-3′), Hsp27-2 (5′-UCGAGGCCCUGUAACUUG-3′), and Hsp27-3 (5′-CAGUAGUUCGGACAAACGAAGA-3′) were designed based on the publicly released Hsp27 DNA sequence and another three oligonucleotides FLIP-1, FLIP-2, and FLIP-3 designed for c-FLIP. The siRNAs were transfected into PC-3 cells with Lipofectamine 2000 (Invitrogen) employing 50 nM in 250 $\mu$L Opti-MEM medium/60 mm culture dish. The transfected cells were allowed to grow for 24, 48, and 72 h at 37°C in a 5% $CO_2$ incubator.

*2.4. Total RNA Extraction, Conventional RT-PCR, and Real-Time RT-PCR.* Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Cells ($5.0 \times 10^5$) were mixed in a test tube with 1 mL TRIzol solution. Prepared RNA was denaturated at 65°C for 15 min in a volume of 30 $\mu$L and cooled on ice for at least 1 min. 2.0 $\mu$g of denatured RNA were then annealed by addition of reaction mixture to a total volume of 20 $\mu$L (4.0 $\mu$L of 5× RT buffer, 10 pmol of primers, 2.0 $\mu$L of 25 mM $MgCl_2$, 2.0 $\mu$L of 10 mM dNTPs, and 0.2 $\mu$L of 1 M DTT in nuclease-free water) and incubated at 42°C for 70 min. The reaction was terminated at 95°C for 5 min, chilled on ice for 5 min and collected by brief centrifugation. To remove RNA, 1 $\mu$L of RNase H was added to each tube followed by incubation at 37°C for 20 min. 1 $\mu$L of cDNA was used for each PCR reaction.

PCR was performed with an SLAN real-time PCR detection system (LG Life Science, Korea) and SYBR Green reagents (Invitrogen, Carlsbad, CA, USA). Specific primers for human GAPDH, Hsp27, and c-FLIP were designed to work in the same cycling conditions (50°C for 2 min to permit uracil *N*-glycosylase cleavage, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min). The specificity of the nucleotide sequences chosen was confirmed by conducting basic local alignment search tool searches. We used 1.0 $\mu$L of the reverse transcriptase product for PCR in a final volume of 25 $\mu$L.

*2.5. Western Blot Analysis.* Preparation of total cell lysate and the procedures for Western blot analyses were performed essentially as described previously [16]. The antibody against c-FLIP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for Hsp27 was purchased from Millipore (Millipore, MA, USA). The quantity of the applied protein was normalized with anti-actin polyclonal antibody (Sigma Aldrich Korea, Seoul, Korea).

Samples with equal amounts of protein (20 $\mu$g) from lysates of cultured PC-3 cells were subjected to SDS-PAGE and then transferred to a PVDF filter. The filters were blocked in TBS containing 5% nonfat milk powder at 4°C overnight and then incubated with each of diluted primary antibodies (Actin: 1 : 10,000; Hsp27: 1 : 1,000; c-FLIP: 1 : 2,000; Santa Cruz, CA, USA) for 1 hour.

*2.6. Immunofluorescence and TUNEL Staining.* Cells on coverslips were rinsed with 1× phosphate-buffered saline (PBS) and then fixed with ice-cold methanol for 15 min. Samples were further permeabilized with PBS containing 0.025% Triton-X detergent (1× PBS-TX) for 10 min and blocked with 3% BSA in 1× PBS for 30 min. Cells were incubated with each of the primary antibodies (Hsp27: 1 : 100; c-FLIP: 1 : 50; Santa Cruz, CA, USA) for 1 hour at room temperature. Cells were washed 3 times for 5 mins with 1× PBS-TX and then incubated with green fluorescent- (FITC-) conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, Santa Cruz, CA, USA). Nuclei were counterstained with Hoechst 33258 (Sigma Chemical, St. Louis, MO, USA).

For TUNEL assays, fixed cells were incubated with an equilibrium buffer for 5 min using the in situ apoptosis detection kit, Fluorescein (ApopTag; Roche, BMS), and then treated in reaction buffer with 10 units of terminal deoxynucleotidyl transferase and 1 unit of deoxyuridine triphosphate-digoxigenin at 37°C for 1 hour. The reaction was terminated by adding stop/wash buffer and then washed twice with Tris buffer. Antidigoxigenin-FITC was added and reacted at 37°C for 30 min. After washing with distilled water, nuclei were counterstained with Hoechst 33258 (Sigma Chemical, St. Louis, MO, USA), and apoptosis in the cells was observed under a fluorescent microscope. Cells with green fluorescent (FITC) colored nuclei were considered apoptotic.

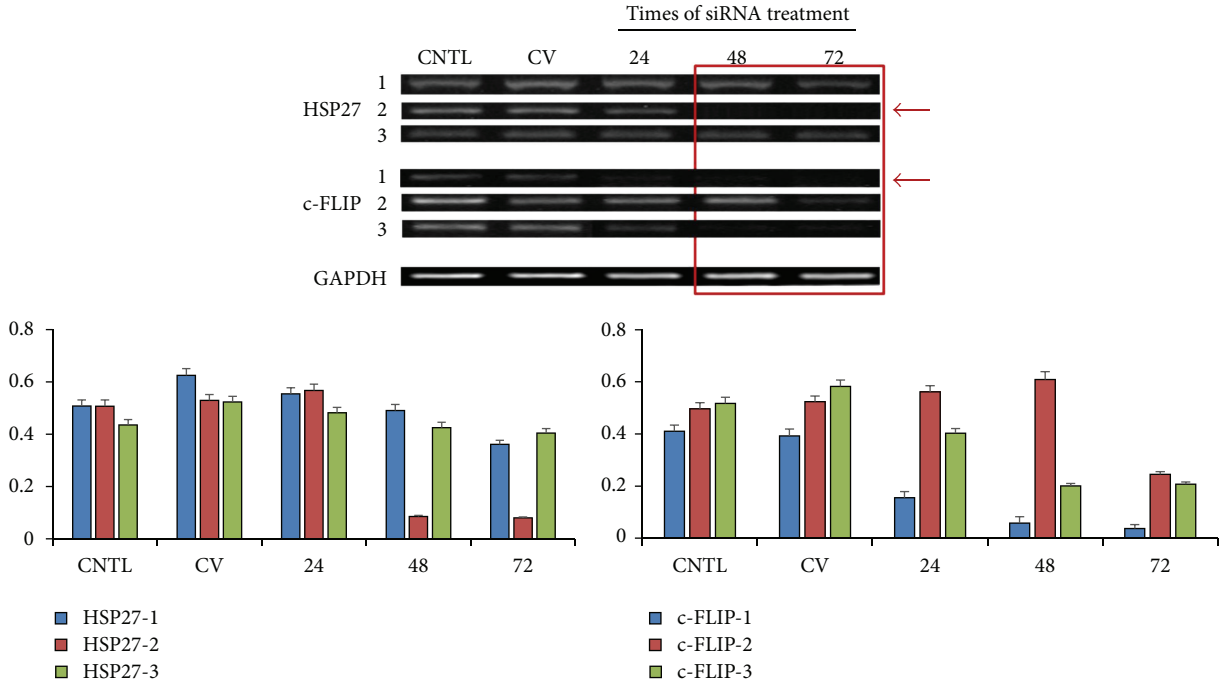


Figure 1: RT-PCR bands show effective silencing of the heat shock protein 27 (Hsp27) and c-FLIP gene expression in PC-3 cells after small interfering RNA (siRNA) treatment.

For quantifying apoptotic cells, apoptotic and total cells were counted in 5 random fields scoring between 300 and 500 cells, and the numbers of apoptotic cells were expressed as percentages of the total cell population. Immunofluorescent staining slides and TUNEL staining slides were observed with microscope (TE-300, Nikon, Japan).

*2.7. Statistical Analysis.* Data are expressed as mean ± SD or median (interquartile range).

## 3. Results

*3.1. Assessment for Hsp27 and c-FLIP RNA Interference with siRNAs in PC-3 Cells.* For downregulation of Hsp27 expression in PC-3 cells, three different Hsp27-specific siRNAs (Hsp27-1, Hsp27-2, and Hsp27-3) were used for transfection studies. As a control, cells were transfected with siRNA against scrambled sequence. To determine the efficiency of the downregulation of Hsp27 expression in PC-3 cells, mRNA levels of Hsp27 were counted by RT-PCR. Forty-eight hours after transfection, Hsp27-2 siRNA downregulated Hsp27 mRNA level to approximately 17% of control level. The downregulation of Hsp27 expression caused by Hsp27-2 siRNA-mediated silencing was maintained until 72 hours (Figure 1).

Similar to Hsp27, three kinds of siRNAs (c-FLIP-1, c-FLIP-2, and c-FLIP-3) were used for c-FLIP silencing in PC-3 cells. c-FLIP-1 siRNA downregulated c-FLIP mRNA level to approximately 14% of control level after 48 hours of transfection. Among three siRNAs, The c-FLIP-1 siRNA-mediated silencing decreased c-FLIP mRNA expression until 72 hours (Figure 1).

Endogeneous expression of Hsp27 in Hsp27-2 siRNA-transfected PC-3 cells was reduced approximately to 14.6% of control level after 48 hours after transfection when measured by Western blot analysis with an anti-Hsp27 antibody (Figure 2). Likewise, Western blot analysis for anti-c-FLIP antibody also revealed the reduction of endogeneous c-FLIP expression in c-FLIP-1 siRNA-transfected PC-3 cells in 18.7% after 48 hours and maintained until 72 hours (Figure 2).

*3.2. TUNEL Analysis.* Figure 3 shows the results of Hoechst and TUNEL fluorescent staining for nuclear morphology and patterns of apoptosis in Hsp27 and c-FLIP gene silenced PC-3 cells when treated with doxazosin (1, 10, and 25 $\mu$M) for 24 hours. Nuclear condensation and fragmentation, characteristic findings of apoptosis, were found in TUNEL-positive cells.

In the cells without siRNA transfection, the number of TUNEL-positive cells was minimal under 1 $\mu$M of doxazosin treatment, but it increased gradually in a dose-dependent manner, and finally a significant number of apoptotic bodies were observed under 25 $\mu$M of doxazosin treatment.

Compared to the nonsilenced cells, in the cells transfected with either siRNA targeting Hsp27 or c-FLIP genes, the numbers of TUNEL-positive cells were increased in all concentrations of doxazosin. And when they were transfected with both siRNAs targeting Hsp27 and c-FLIP gene together, the number of TUNEL-positive cells was increased more significantly in all concentrations of doxazosin. In this group

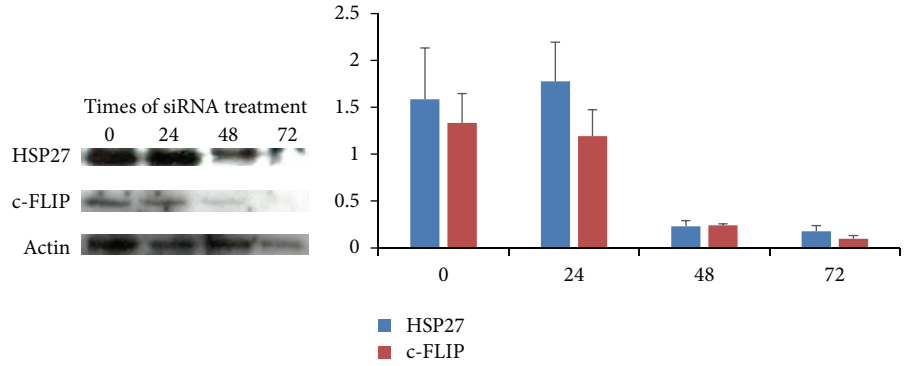


Figure 2: Immunoblotting shows effective silencing of the heat shock protein 27 (Hsp27) and c-FLIP in PC-3 cells after siRNA treatment.

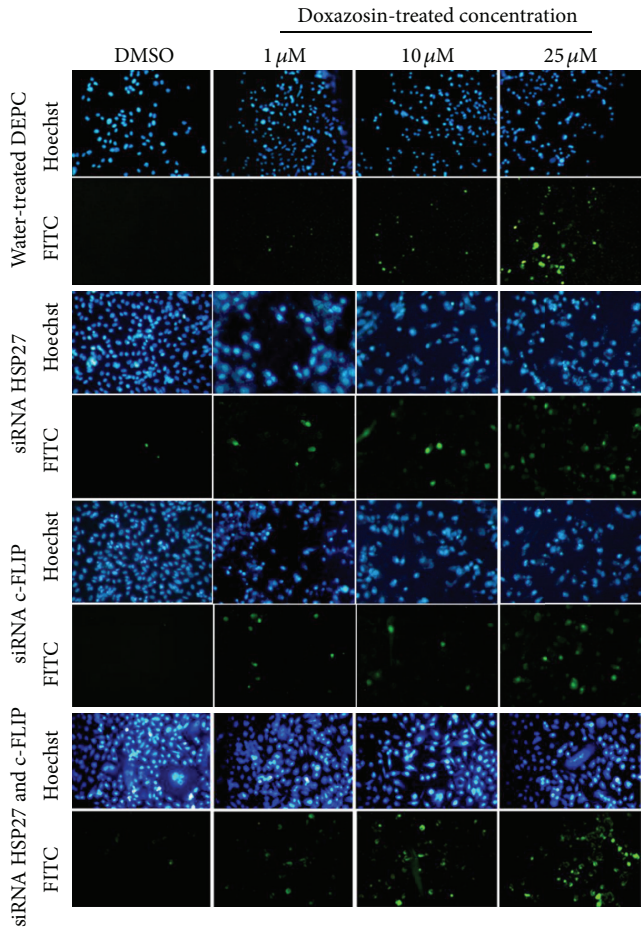


Figure 3: In situ detection of apoptotic cells in siRNA (Hsp27, c-FLIP, dual) transfected PC-3 cells after 48 hours at each dosage of doxazosin treatment (1, 10, and 25 $\mu$M). In situ detection of apoptotic cells in prostate cancer cells was performed by 3′-end labeling with digoxigenin-dUTP using terminal transferase.

of cells, TUNEL-positive cells were visible quite a lot even with 1 $\mu$M of doxazosin treatment.

Spontaneous apoptotic index was 5% under single gene silencing of Hsp27 or c-FLIP and 7% under dual silencing of Hsp27 and c-FLIP genes together. When doxazosin

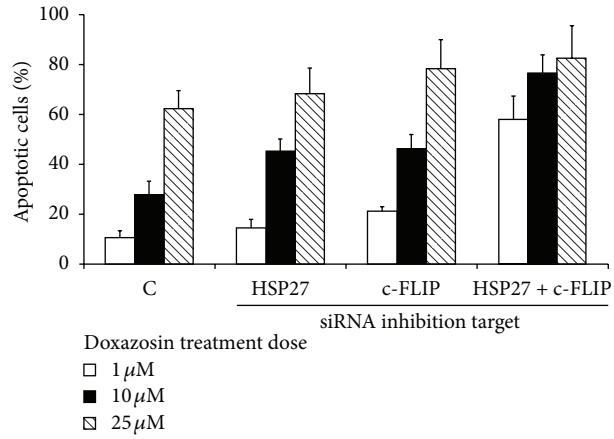


Figure 4: Apoptotic indices in siRNA (Hsp27, c-FLIP) transfected cells after 48 hours at each dosage of doxazosin treatment (1, 10, and 25 $\mu$M).

treatment was added, apoptotic indices increased in the dose-dependent manner (1, 10, and 25 $\mu$M): nonsilencing 10, 27, and 52%; Hsp27 silencing: 14, 35, and 68%; c-FLIP silencing: 21, 46, and 78%; dual gene silencing: 38, 76, and 92% (Figure 4). Annexin V staining showed similar findings (data not shown).

*3.3. Cross-Checking for Hsp27 and c-FLIP RNA Interference with siRNA for PC-3.* The interaction between 2 factors is investigated by observing the expression of one factor under silencing of the other factor. When Hsp27 is silenced successfully, c-FLIP gene expression was suppressed, and when c-FLIP was inhibited by siRNA transfection, Hsp27 gene expression was also downregulated (Figure 5). Similar findings were observed in protein level. Western blot analysis showed that when c-FLIP was inhibited by siRNA transfection, Hsp27 protein expression was downregulated, and protein expression of c-FLIP was suppressed when Hsp27 gene was silenced. Addition of 1 $\mu$M of doxazosin enhanced downregulation of c-FLIP protein expression induced by Hsp27 gene silencing (37.5% → 16.4%) (Figure 6).

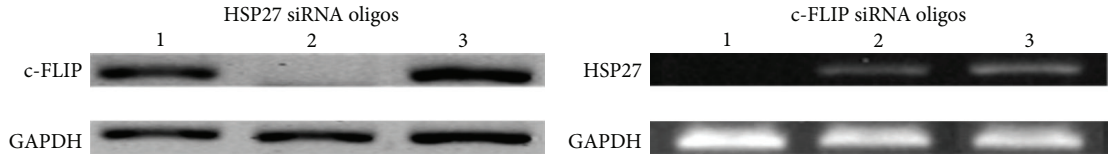


Figure 5: RT-PCR band of small interfering RNA (siRNA) treatment of the heat shock protein 27 (Hsp27) and c-FLIP after 48 hours in PC-3 cells.

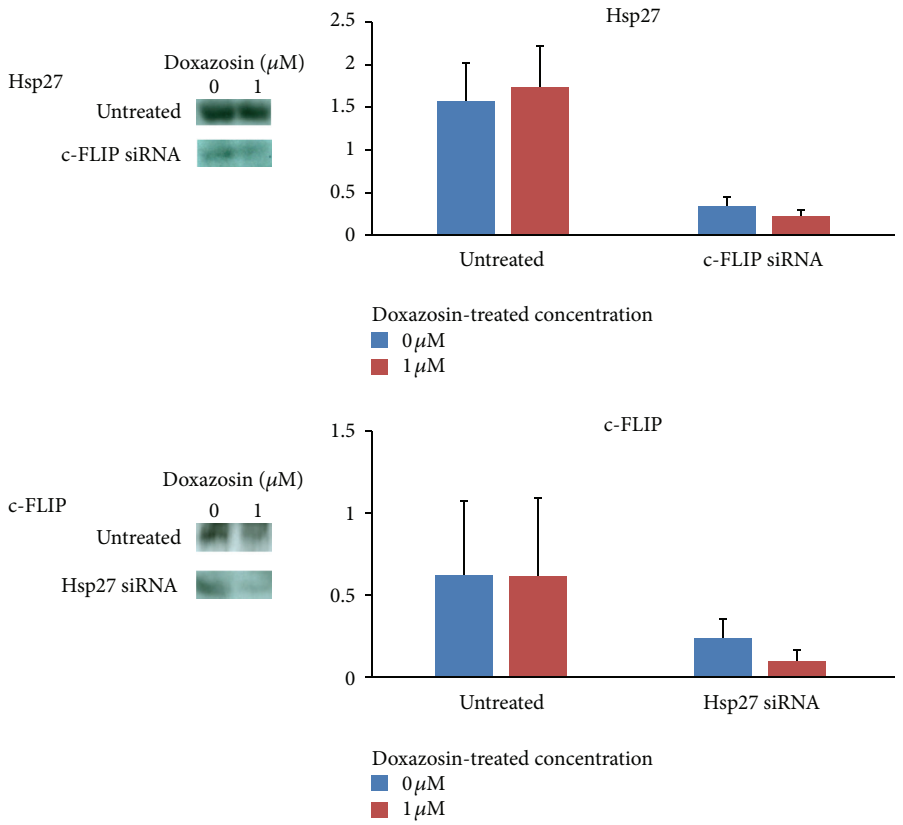


Figure 6: Electrophoretogram of immunoblot for Hsp27 and c-FLIP expressions at the PC-3 cells.

## 4. Discussion

The experimental dose of doxazosin, 10–25 $\mu$M, is relatively higher than the serum concentration of patients treated with doxazosin for their lower urinary tract symptoms (LUTS) [24]. Thus, doxazosin at clinical dose cannot induce significant apoptosis in the patients with prostate cancer. Therefore, the findings of new mechanisms showing the induction of apoptosis at very low concentrations of doxazosin may provide an evidence to put doxazosin as a new drug candidate for treatment of prostate cancer [25].

Commonly used siRNA introduction techniques are either direct introduction by transfection or introduction via plasmids that express short-hairpin RNA (shRNA) precursors of siRNA [26]. In this study, siRNA was introduced by the direct transfection way. We selected a proper oligo which could knock out Hsp27 or c-FLIP gene after 48 hours of siRNA transfection.

Rocchi et al. reported the effect of synthetic siRNA targeting Hsp27 in PC-3 and LNCaP cells. According to their reports, 1 nM of siRNA was effective to downregulate Hsp27 in mRNA and protein levels, resulting in 2.4–4-fold increase of apoptotic rates and 40%–76% inhibition of cell growth. Characteristic cleavage of caspase-3 was also observed [27].

Day et al. reported c-FLIP knockdown in MCF-7 breast cancer cells. They observed knockdown of c-FLIP gene with siRNA transfection triggering spontaneous apoptosis and inducing FADD-mediated and DR-5-mediated apoptosis. They addressed c-FLIP$_L$ not c-FLIPs for having a key role in preventing spontaneous death signaling and suggested c-FLIP$_L$ as a therapeutic target for breast cancer [16]. Similarly, in a report on colorectal cancer cells, Longley et al. observed that siRNA targeting c-FLIP$_L$ synergistically enhanced chemotherapy-induced apoptosis [28].

In our present study, TUNEL-positive apoptotic cells increased twice with each siRNA transfection and increased over 60% after a dual silencing of 2 genes. After single gene silencing of Hsp27, apoptotic index was remarkably increased in 10 and 25 $\mu$M of doxazosin treatment condition. Similarly, single silencing of c-FLIP gene enhanced doxazosin-induced

apoptosis, but the degree of apoptosis was little higher in c-FLIP group compared to Hsp27 group. It can be speculated that while both factors are working as antiapoptotic factors significantly, c-FLIP plays bigger role in resisting doxazosin-induced apoptosis in PC-3 cells.

siRNA technology can be used in combined knockdown of two genes involved in carcinogenesis or cancer progression via dual silencing. Kaulfu$\beta$ and colleagues reported that dual silencing of insulin-like growth factor receptor and epidermal growth factor receptor resulted in an increased apoptosis rate and inhibition of cell proliferation in colorectal cancer cells. Dual silencing is technically feasible and effective in augmenting biologic effect in laboratory level. However, it has not yet been widely established. The studies using this method are limited to colorectal and breast cancers and are also limited to the region of growth factor. As far as we know, there has been no study blocking two different antiapoptotic proteins in prostate cancer cells.

Both Hsp27 and c-FLIP have been known as strong antiapoptotic mediators. While c-FLIP manifests its role mainly in extrinsic apoptotic pathway, Hsp27 does mainly in mitochondrial pathway. For this reason, we planned to knock out these 2 factors which have different antiapoptotic mechanisms together. We observed much more amount of apoptotic bodies when these 2 factors are blocked together by siRNA technology than individual silencing of Hsp27 or c-FLIP alone. Furthermore, this effect could be seen in low concentration of 1 $\mu$M of doxazosin. These show that PC-3 cells which could have resisted against apoptosis with the help of 2 survival factors became susceptible to doxazosin treatment when Hsp27 and c-FLIP are effectively knocked down together. If applied to clinical situations, this result suggests that multiple block of several antiapoptotic factors which are overexpressed and helped cancer cells to resist to treatment induced apoptosis can augment therapeutic effect even in very low concentration of the drug.

We also observed that both factors interacted with each other. c-Flip knockout downregulated the expression of Hsp27, and similarly, silencing against Hsp27 decreased the expression of c-FLIP in RT-PCR study. These findings suggest that antiapoptotic functions of c-FLIP and Hsp27 are closely related even when the main pathways are different. We previously reported that siRNA targeting androgen receptor reversed the expression of Hsp27, GRP78, clusterin, and c-FLIP in long-term cultured androgen-independent LNCaP cell lines [28, 29]. Through these observations, we can speculate that prostate cancer cells have their own peculiar features of antiapoptotic mechanisms that include close interaction between androgen receptor and several survival factors.

## 5. Conclusions

Dual silencing is technically feasible, and dual silencing of c-Flip and Hsp27 enhances apoptosis even under 1 $\mu$M, rather than low concentration, of doxazosin in PC-3 cells. This suggests a new strategy of multiple knockout of anti-apoptotic and survival factors in the treatment of late-stage prostate cancer refractory to conventional therapy. We also preliminarily observed that there was interaction between c-FLIP and Hsp27 expression. Further studies revealing detailed interactions between important survival factors and androgen receptor can make another basis in reinforcing therapeutic armaments combating fatal advanced prostate cancer.

## Conflict of Interests

All authors confirm that they have no conflict of interests.

## Acknowledgment

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## References

[1] I. F. Tannock, R. De Wit, W. R. Berry et al., "Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer," *New England Journal of Medicine*, vol. 351, no. 15, pp. 1502–1512, 2004.

[2] D. P. Petrylak, C. M. Tangen, M. H. A. Hussain et al., "Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer," *New England Journal of Medicine*, vol. 351, no. 15, pp. 1513–1520, 2004.

[3] P. Cheetham and D. P. Petrylak, "Tubulin-targeted agents including docetaxel and cabazitaxel," *Cancer Journal*, vol. 19, no. 1, pp. 59–65, 2013.

[4] M. N. Stein, S. Goodin, and R. S. DiPaola, "Abiraterone in prostate cancer: a new angle to an old problem," *Clinical Cancer Research*, vol. 18, no. 7, pp. 1848–1854, 2012.

[5] J. Bai, J. Sui, A. Demirjian, C. M. Vollmer Jr., W. Marasco, and M. P. Callery, "Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro," *Cancer Research*, vol. 65, no. 6, pp. 2344–2352, 2005.

[6] S. D. Catz and J. L. Johnson, "BCL-2 in prostate cancer: a minireview," *Apoptosis*, vol. 8, no. 1, pp. 29–37, 2003.

[7] Y. H. Youm, H. Yang, Y.-D. Yoon, D.-Y. Kim, C. Lee, and T. K. Yoo, "Doxazosin-induced clusterin expression and apoptosis in prostate cancer cells," *Urologic Oncology*, vol. 25, no. 6, pp. 483–488, 2007.

[8] P. Rocchi, A. So, S. Kojima et al., "Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer," *Cancer Research*, vol. 64, no. 18, pp. 6595–6602, 2004.

[9] A. Glaessgen, S. Jonmarker, A. Lindberg et al., "Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer," *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 116, no. 10, pp. 888–895, 2008.

[10] A. R. Safa, T. W. Day, and C.-H. Wu, "Cellular FLICE-like inhibitory protein (C-FLIP): a novel target for cancer therapy," *Current Cancer Drug Targets*, vol. 8, no. 1, pp. 37–46, 2008.

[11] L. Pootrakul, R. H. Datar, S.-R. Shi et al., "Expression of stress response protein Grp78 is associated with the development of castration-resistant prostate cancer," *Clinical Cancer Research*, vol. 12, no. 20, pp. 5987–5993, 2006.

[12] A. Zoubeidi, K. Chi, and M. Gleave, "Targeting the cytoprotective chaperone, clusterin, for treatment of advanced cancer," *Clinical Cancer Research*, vol. 16, no. 4, pp. 1088–1093, 2010.

[13] D. Lanneau, A. de Thonel, S. Maurel, C. Didelot, and C. Garrido, "Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27," *Prion*, vol. 1, no. 1, pp. 53–60, 2007.

[14] P. A. Cornford, A. R. Dodson, K. F. Parsons et al., "Heat shock protein expression independently predicts clinical outcome in prostate cancer," *Cancer Research*, vol. 60, no. 24, pp. 7099–7105, 2000.

[15] S. L. Lee, E. K. Kim, S. S. Kim, S. H. Uh, K. C. Cha, and T. K. Yoo, "Expression of heat shock protein 27 according to Gleason score and pathologic stage of prostate cancer," *Korean Journal of Urology*, vol. 50, no. 6, pp. 547–552, 2009.

[16] T. W. Day, S. Huang, and A. R. Safa, "c-FLIP knockdown induces ligand-independent DR5-, FADD-, caspase-8-, and caspase-9-dependent apoptosis in breast cancer cells," *Biochemical Pharmacology*, vol. 76, no. 12, pp. 1694–1704, 2008.

[17] X. Zhang, T.-G. Jin, H. Yang, W. C. Dewolf, R. Khosravi-Far, and A. F. Olumi, "Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer," *Cancer Research*, vol. 64, no. 19, pp. 7086–7091, 2004.

[18] P. Korkolopoulou, A. A. Saetta, G. Levidou et al., "c-FLIP expression in colorectal carcinomas: association with Fas/FasL expression and prognostic implications," *Histopathology*, vol. 51, no. 2, pp. 150–156, 2007.

[19] P. Korkolopoulou, A. Goudopoulou, G. Voutsinas et al., "c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations," *Urology*, vol. 63, no. 6, pp. 1198–1204, 2004.

[20] T. W. Day, A. L. Sinn, S. Huang, K. E. Pollok, G. E. Sandusky, and A. R. Safa, "C-FLIP gene silencing eliminates tumor cells in breast cancer xenografts without affecting stromal cells," *Anticancer Research*, vol. 29, no. 10, pp. 3883–3886, 2009.

[21] S. Kaulfuß, P. Burfeind, J. Gaedcke, and J.-G. Scharf, "Dual silencing of insulin-like growth factor-I receptor and epidermal growth factor receptor in colorectal cancer cells is associated with decreased proliferation and enhanced apoptosis," *Molecular Cancer Therapeutics*, vol. 8, no. 4, pp. 821–833, 2009.

[22] C. M. Benning and N. Kyprianou, "Quinazoline-derived $\alpha$1-adrenoceptor antagonists induce prostate cancer cell apoptosis via an $\alpha$1-adrenoceptor-independent action," *Cancer Research*, vol. 62, no. 2, pp. 597–602, 2002.

[23] J. B. Garrison and N. Kyprianou, "Doxazosin induces apoptosis of benign and malignant prostate cells via a death receptor-mediated pathway," *Cancer Research*, vol. 66, no. 1, pp. 464–472, 2006.

[24] N. Kyprianou, T. B. Vaughan, and M. C. Michel, "Apoptosis induction by doxazosin and other quinazoline $\alpha$1-adrenoceptor antagonists: a new mechanism for cancer treatment?" *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 380, no. 6, pp. 473–477, 2009.

[25] N. Kyprianou, "Doxazosin and terazosin suppress prostate growth by inducing apoptosis: clinical significance," *Journal of Urology*, vol. 169, no. 4, pp. 1520–1525, 2003.

[26] V. N. Kim, "RNA interference in functional genomics and medicine," *Journal of Korean Medical Science*, vol. 18, no. 3, pp. 309–318, 2003.

[27] P. Rocchi, E. Beraldi, S. Ettinger et al., "Increased Hsp27 after androgen ablation facilitates androgen-independent progression in prostate cancer via signal transducers and activators of transcription 3-mediated suppression of apoptosis," *Cancer Research*, vol. 65, no. 23, pp. 11083–11093, 2005.

[28] D. B. Longley, T. R. Wilson, M. McEwan et al., "c-FLIP inhibits chemotherapy-induced colorectal cancer cell death," *Oncogene*, vol. 25, no. 6, pp. 838–848, 2006.

[29] S. S. Kim, H. J. Cho, J. Y. Kang, H. K. Kang, and T. K. Yoo, "Inhibition of androgen receptor expression with small interfering RNA enhances cancer cell apoptosis by suppressing survival factors in androgen insensitive, late stage LNCaP cells," *The Scientific World Journal*, vol. 2013, Article ID 519397, 8 pages, 2013.

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*Clinical Study*

# Secondary Circulating Prostate Cells Predict Biochemical Failure in Prostate Cancer Patients after Radical Prostatectomy and without Evidence of Disease

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*Introduction*. Although 90% of prostate cancer is considered to be localized, 20%–30% of patients will experience biochemical failure (BF), defined as serum PSA >0.2 ng/mL, after radical prostatectomy (RP). The presence of circulating prostate cells (CPCs) in men without evidence of BF may be useful to predict patients at risk for BF. We describe the frequency of CPCs detected after RP, relation with clinicopathological parameters, and association with biochemical failure. *Methods and Patients*. Serial blood samples were taken during followup after RP, mononuclear cells were obtained by differential gel centrifugation, and CPCs identified using standard immunocytochemistry using anti-PSA monoclonal antibodies. Age, pathological stage (organ confined, nonorgan confined), pathological grade, margin status (positive, negative), extracapsular extension, perineural, vascular, and lymphatic infiltration (positive, negative) were compared with the presence/absence of CPCs and with and without biochemical failure. Kaplan Meier methods were used to compare the unadjusted biochemical failure free survival of patients with and without CPCs. *Results*. 114 men participated, and secondary CPCs were detected more frequently in patients with positive margins, extracapsular extension, and vascular and lymphatic infiltration and were associated with biochemical failure independent of these clinicopathological variables, and with a shorter time to BF. *Conclusions*. Secondary CPCs are an independent risk factor associated with increased BF in men with a PSA <0.2 ng/mL after radical prostatectomy, but do not determine if the recurrence is due to local or systemic disease. These results warrant larger studies to confirm the findings.

## 1. Introduction

In the PSA era more than 90% of prostate cancer cases are considered to be localized at the time of diagnosis; however, 20%–30% of these patients will experience biochemical failure, usually in the first two years after surgery [1, 2]. Biochemical failure may occur as late as 10 to 15 years after primary treatment, with a mean time of 8 years from biochemical failure to the appearance of clinical metastasis [1]. This suggests the persistence of tumor cells in a state of either complete or near dormancy prior to metastatic progression. It has been reported that disseminated tumor cells in bone marrow predict biochemical failure after radical prostatectomy [3]. Disseminated tumor cells (DTCs) in bone marrow aspirates were detected in 57% of patients without evidence of disease after radical prostatectomy and detected in 45% of patients 5 years after surgery. These patients positive for DTCs in bone marrow aspirates had a nearly 7-fold

increased risk of biochemical failure compared with patients DTC negative [3]. Although the risks of complications after bone marrow aspiration are estimated to be approximately 0.4% [4], the use of circulating prostate cells in blood would be easier to implement. It has been recently published that circulating prostate cells (CPCs) are phenotypically identical to DTCs and that both represent circulating cells in two different tissue compartments in patients with prostate cancer and not true micrometastasis [5]. Early dissemination of cancer cells regardless of stage, grade, or tumor volume has been previously reported; dissemination first occurs to the neurovascular structures and then onto the circulation [6]. The majority of these cells will be eliminated by host defense mechanisms; however a small number will implant in distant tissues, survive, and in time proliferate. These cells will not be eradicated by radical prostatectomy and may later be detected in the circulation, secondary CPCs.

The purpose of this study was to describe the prevalence of secondary CPCs after radical prostatectomy and determine if this information would be clinically relevant and if there was an association with biochemical failure.

## 2. Methods and Patients

*2.1. Patient Selection.* From January 2009 to December 2011 blood samples from consecutive prostate cancer patients were prospectively collected for the purpose of detecting CPCs and evaluating whether these cells were correlated with clinical outcomes. All patients who had undergone radical prostatectomy at the author's institution and all those seen during followup were invited to participate. Samples were taken from men at least three months after surgery and considered to be without evidence of disease. This was defined as being bone scan negative and a serum PSA <0.20 ng/mL. A group of men with a serum PSA of 0.2–1.0 ng/mL and bone scan negative was selected to represent men with biochemical failure. All samples were obtained after written informed consent and collected using protocols approved by the local ethics committee.

*2.2. Sample Collection and Cell Enrichment.* 8 mL of venous blood was collected in tubes containing EDTA (Beckson-Vacutainer). Mononuclear cells were obtained using gel differential centrifugation using Histopaque 1,077 (Sigma-Aldrich) at room temperature according to manufacturer's instructions and finally washed 3 times in phosphate-buffered saline pH 7.4 (PBS). The pellet was resuspended in 100 $\mu$L of autologous plasma and 25 $\mu$L used to prepare each slide (silanized DAKO, USA). The slides were air-dried for 24 hours and finally fixed in a solution of 70% ethanol, 5% formaldehyde, and 25% PBS for 5 minutes and then washed 3 times with PBS.

*2.3. Identification of CPCs.* Slides were processed within 1 hour of fixation and incubated with anti-PSA clone 28A4 (Novocastra Laboratory, UK) in a concentration of 2.5 $\mu$g/mL for 1 hour at room temperature and identified using a detection system based on alkaline phosphatase-antialkaline phosphatase (LSAB2 DAKO, USA) with new fuchsin as the chromogen. To permit the rapid identification of positive cells there was no counterstaining with Mayer's hematoxylin. Levamisole (DAKO, USA) was used as an inhibitor of endogenous alkaline phosphatase. Positive and negative controls were processed in the same way.

Definition of secondary CPCs using the criteria of ISHAGE was used to identify immunostained cells (7), a cell positive for PSA with a nucleus (Figures 1(a) and 1(b)). Samples were analyzed at low power and photographed at a magnification of 400x using a digital camera, Samsung Digimax D73, and processed with the Digimax program for Windows 98. The immunocytochemical evaluation was performed by a single person, blinded to the clinical details using a coded system.

Table 1: Demographic details of the study population.

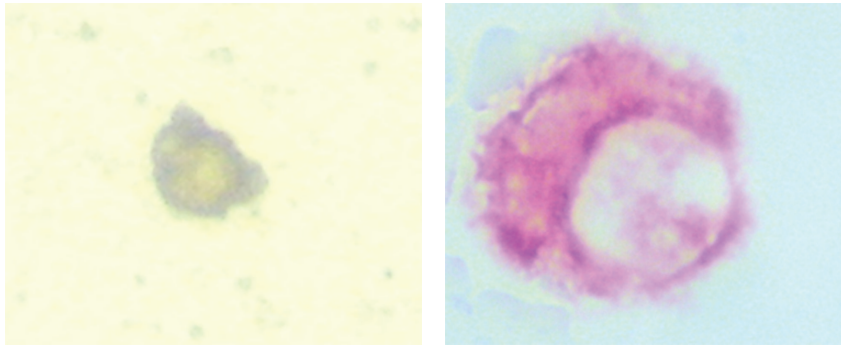
| | Group 1 | Group 2 | Group 3 | Total |
|---|---|---|---|---|
| No. of patients | 28 | 64 | 22 | 114 |
| Initial stage | | | | |
| 1 | 9 | 9 | 0 | |
| 2 | 16 | 33 | 11 | |
| 3 | 3 | 22 | 11 | |
| Median Gleason score (IQR) | 6 (5-6) | 6 (5-6) | 6 (5-6) | |
| Time from surgery (years) | 3.8 ± 1.6 | 3.2 ± 2.9 | 5.6 ± 3.6 | |

*2.4. Statistical Methods.* Descriptive statistics were used to compare demographic and disease characteristics of patients with and without biochemical failure. Univariate comparisons were tested using chi-squared and Kaplan Meier methods were used to compare the unadjusted free from biochemical failure of patients with and without CPCs detected. Age, pathological stage (organ confined, nonorgan confined), pathological grade, margin status (positive, negative), extracapsular extension (positive, negative), and perineural, vascular, and lymphatic infiltration (positive, negative) were compared with the presence/absence of CPCs and with and without biochemical failure.

Because the time between radical prostatectomy and the blood sampling was not standardized, two separate models were considered. In the first model, the time under observation started at the date of radical prostatectomy. In the second the time under observation started at the time of blood sampling after surgery. Patients who did not experience biochemical failure were censored at the date of last followup.

## 3. Results

114 men with a mean age of 71.5 ± SD 8.2 years participated. Table 1 shows the distribution of patients according to PSA levels, pathological stage at diagnosis, and median time from surgery to blood sampling. Men in Group 1 had significantly less pT3 disease ($P = 0,04$ chi-squared) than Group 2.



(a) (b)

Figure 1: (a) Leucocyte (PSA negative). (b) CPC PSA positive.

Table 2: Secondary CPC detection and association with clinical parameters.

| Clinical parameter | CPC (+) | CPC (−) | |
|---|---|---|---|
| Margin (+) | 15 | 7 | $P = 0.003$ |
| Margin (−) | 23 | 47 | |
| Capsule (+) | 24 | 17 | $P = 0.003$ |
| Capsule (−) | 14 | 37 | |
| Perineural (+) | 27 | 29 | $P = 0.09$ |
| Perineural (−) | 11 | 25 | |
| Vascular (+) | 15 | 3 | $P = 0.00005$ |
| Vascular (−) | 23 | 51 | |
| Lymphatic (+) | 13 | 4 | $P = 0.001$ |
| Lymphatic (−) | 25 | 50 | |
| Gleason 4 | 1 | 11 RR 1.00 | |
| Gleason 5 + 6 | 31 | 23 RR 7.41 | $P = 0.015$ for trends |
| Gleason 7 | 8 | 11 RR 4.00 | |
| Gleason 8 + 9 | 8 | 1 RR 44.00 | |

Table 3: Biochemical failure and association with clinical parameters.

| Clinical parameter | BF (+) | BF (−) | |
|---|---|---|---|
| Margin (+) | 8 | 14 | $P = 0.97$ |
| Margin (−) | 22 | 48 | |
| Capsule (+) | 14 | 24 | $P = 0.53$ |
| Capsule (−) | 16 | 38 | |
| Perineural (+) | 25 | 30 | $P = 0.001$ |
| Perineural (−) | 5 | 32 | |
| Vascular (+) | 10 | 8 | $P = 0.02$ |
| Vascular (−) | 20 | 54 | |
| Lymphatic (+) | 8 | 8 | $P = 0.08$ |
| Lymphatic (−) | 22 | 54 | |
| Gleason 4 | 0 | 12 | |
| Gleason 5 + 6 | 18 | 36 | $P = 0.05$ for trends |
| Gleason 7 | 8 | 11 | |
| Gleason 8 + 9 | 4 | 5 | |

Secondary CPCs were detected in 10/28 (35.7%) men in Group 1, 27/64 (42.2%) in Group 2, and 15/22 (68.2%) in Group 3. There was a significant tendency of increased frequency of CPC detection with increasing serum PSA ($P = 0.002$ chi-squared for tendency) with a relative risk of 1.00, 1.31, and 3.86, respectively.

Secondary CPCs were detected more frequently in patients with positive margins, extracapsular extension, and vascular and lymphatic infiltration but not with perineural infiltration (Table 2). There was a trend with increasing frequency of CPC detection with pathological stage ($P = 0.002$ chi-squared for trends) with a relative risk of 1.00, 3.63 and 10.83 for stages pT1, pT2, and pT3, respectively, and with increasing Gleason score ($P = 0.015$) with a relative risk of 1.00, 7.41, 4.00, and 44.00 for Gleason 4, 5 + 6, 7 and 8 + 9, respectively.

*3.1. Analysis of Biochemical Failure in Groups 1 and 2.* 7/28 (25.0%) of men in Group 1 and 23/64 (35.9%) of men in Group 2 experienced biochemical failure within the study period ($P = 0.37$ chi-squared). Comparing men with and without biochemical failure, there were no significant differences in the number of patients with margins positive 9/22 versus 22/70 ($P = 0.64$ chi-squared) or capsule compromised 14/38 versus 16/54 ($P = 0.53$ chi-squared). Biochemical failure was more frequent in men with perineural infiltration 25/55 versus 5/37 ($P = 0.001$ chi-squared) and vascular infiltration 10/18 versus 20/74 ($P = 0.021$ chi-squared) but not with lymphatic infiltration 8/16 versus 22/77 ($P = 0.08$ chi-squared).

There was a trend of increasing biochemical failure with increasing Gleason score, comparing Gleason 4, Gleason 5 + 6, Gleason 7, and Gleason 8 + 9, ($P = 0.05$ chi-squared for trends), with a relative risk of 1.00, 6.00, 8.70, and 9.60, respectively (Table 3).

*3.2. Association of CPC Status and Clinicopathological Parameters with Biochemical Failure.* Incorporating the detection of CPCs with the pathological parameters showed different results. 25/38 (65.8%) men CPC positive experienced biochemical failure in comparison with 5/56 (8.9%) of men CPC negative ($P = 0.0001$ chi-squared).

Table 4: (a) Association of CPC status and margin status with biochemical failure. (+) positive (−) negative, (b) Association of CPC status and capsule status with biochemical failure, (c) Association of CPC status and perineural infiltration with biochemical failure, (d) Association of CPC status and vascular infiltration with biochemical failure, (e) Association of CPC status and lymphatic infiltration with biochemical failure.

(a)

| | Con biochemical failure | Total | RR |
|---|---|---|---|
| (a) Margin (+) CPC (+) | 9 | 15 | 16.5 |
| (b) Margin (+) CPC (−) | 1 | 7 | 1.83 |
| (c) Margin (−) CPC (+) | 17 | 22 | 37.4 |
| (d) Margin (−) CPC (−) | 4 | 48 | 1.0 |

(b)

| | Con biochemical failure | Total | RR |
|---|---|---|---|
| (a) Capsule (+) CPC (+) | 13 | 22 | 12.6 |
| (b) Capsule (+) CPC (−) | 1 | 16 | 0.58 |
| (c) Capsule (−) CPC (+) | 12 | 15 | 35.0 |
| (d) Capsule (−) CPC (−) | 4 | 39 | 1.0 |

(c)

| | Con biochemical failure | Total | RR |
|---|---|---|---|
| (a) PN (+) CPC (+) | 20 | 25 | 100.0 |
| (b) PN (+) CPC (−) | 5 | 30 | 5.0 |
| (c) PN (−) CPC (+) | 5 | 11 | 20.9 |
| (d) PN (−) CPC (−) | 0 | 26 | 1.0 |

(d)

| | Con biochemical failure | Total | RR |
|---|---|---|---|
| (a) V (+) CPC (+) | 10 | 16 | 17.0 |
| (b) V (+) CPC (−) | 0 | 2 | 3.4 |
| (c) V (−) CPC (+) | 15 | 18 | 51.0 |
| (d) V (−) CPC (−) | 5 | 56 | 1.0 |

(e)

| | Con biochemical failure | Total | RR |
|---|---|---|---|
| (a) L (+) CPC (+) | 8 | 13 | 15.0 |
| (b) L (+) CPC (−) | 0 | 3 | 3.1 |
| (c) L (−) CPC (+) | 17 | 25 | 20.0 |
| (d) L (−) CPC (−) | 5 | 52 | 1.0 |

(f)

| | CPC (+) | BF | CPC (−) | BF |
|---|---|---|---|---|
| Gleason 4 | 1 | 0 | 11 | 0 |
| Gleason 5 + 6 | 31 | 16 | 23 | 2 |
| Gleason 7 | 8 | 5 | 11 | 3 |
| Gleason 8 + 9 | 8 | 4 | 1 | 0 |
| Total | | | | |

*3.2.1. CPC and Margin Status (Table 4(a)).* Men CPC (+) margin (+) were more likely to experience biochemical failure than men CPC (−) margin (+), 9/15 versus 0/7 ($P = 0.022$ chi-squared); likewise men CPC (+) margin (−) were more likely to experience biochemical failure than men CPC (−) margin (−), 17/22 versus 4/50 ($P = 0.0001$ Chi-squared) (Table 6). Comparing CPC (+) margin (+) with CPC (+) margin (−) there was no significant difference ($P = 0.16$ chi-squared); similarly there was no difference between CPC (−) margin (+) and CPC (−) margin (−) ($P = 1.00$ Fisher two-tailed).

*3.2.2. CPC and Extracapsular Extension (Table 4(b)).* Men CPC (+) capsule (+) were more likely to experience biochemical failure than men CPC (−) capsule (+), 13/33 versus 1/16 ($P = 0.0008$ Fisher two-tailed); likewise men CPC (+) capsule (−) were more likely to experience biochemical failure than men CPC (−) capsule (−) ($P = 0.0001$, Fisher two-tailed). Comparing CPC (+) capsule (+) with CPC (+) margin (−) there was no significant difference ($P = 0.47$); equally there was no significant difference between CPC (−) capsule (+) with CPC (−) capsule (−) ($P = 1.00$ Fisher two-tailed).

*3.2.3. CPC and Perineural (PN) Infiltration (Table 4(c)).* Men CPC (+) PN (+) were more likely to experience biochemical failure compared with CPC (−) PN (+) 20/25 versus 5/30 ($P = 0.0001$ chi-squared), similarly for men CPC (+) PN (−) versus CPC (−) PN (−), 5/11 versus 0/26 ($P = 0.001$, Fisher two-tailed). Comparing men CPC (+) PN (+) versus CPC (+) PN (−) there was no significant difference ($P = 0.056$ Fisher two-tailed). Similarly for CPC (−) PN (+) versus CPC (−) PN (−) there was no significant difference ($P = 0.055$, Fisher two-tailed).

*3.2.4. CPC and Vascular (V) Infiltration (Table 4(d)).* There was no significant difference in biochemical failure between CPC (+) V (+) and CPC (−) V (+) 10/16 versus 0/2 ($P = 0.18$ Fisher two-tailed); however, men CPC (+) V (−) were more likely to experience biochemical failure than men CPC (−) V (−) 15/18 versus 5/56 ($P = 0.0001$ Fisher two-tailed). Comparing V (+) versus V (−) in men CPC (+) there was no difference ($P = 0.25$ Fisher two-tailed) or comparing V (+) versus V (−) in men CPC (−) ($P = 1.00$ Fisher two-tailed).

*3.2.5. CPC and Lymphatic (L) Infiltration (Table 4(e)).* There was no significant difference in biochemical failure between CPC (+) L (+) and CPC (−) L (+) ($P = 0.2$ Fisher two-tailed); however men CPC (+) L (−) were more likely to experience biochemical failure than men CPC (−) L (−) ($P = 0.0005$ Fisher two-tailed). There were no significant differences in biochemical failure between CPC (+) L (+) versus L (−) or CPC (−) L (+) versus L (−).

*3.2.6. CPC and Gleason Score (Table 4(f)).* There was no significant difference in biochemical failure in relation to the Gleason score in men CPC (+) or in relation to the Gleason score in men CPC (−) nor was there a trend for increasing failure with increasing Gleason score in the two groups, CPC (+) and CPC (−).

*3.2.7. Frequency of Biochemical Failure in CPC Positive and Negative Men with Time from Surgery.* Men CPC positive had a higher frequency of biochemical failure during the first 5 years after surgery; however both CPC positive and negative

Table 5: Kaplan Meyer plot for men without biochemical failure with time after radical prostatectomy.

| | 0 years | 1 year | 2 years | 3 years | 4 years | 5 years | 6 years | 7 years |
|---|---|---|---|---|---|---|---|---|
| CPC (+) | 100% 38/38 | 38/38 | 26/31 | 24/28 | 18/24 | 13/17 | 9/14 | 6/7 |
| CPC (−) | 100% 54/54 | 54/54 | 52/53 | 44/44 | 39/40 | 28/29 | 13/14 | 8/9 |
| | | $P = 1.00$ | $P = 0.02$ | $P = 0.02$ | $P = 0.009$ | $P = 0.06$ | $P = 0.16$ | $P = 1.00$ |

Table 6: Uncensored Kaplan-Meier of men without biochemical failure comparing CPC (+) versus CPC from time of blood sampling.

| | $T = 0$ | $T = 1$ year | $T = 2$ years | $T = 3$ years |
|---|---|---|---|---|
| CPC (+) | 100% (38/38) | 74% (28/38) | 45% (9/20) | 20% (1/5) |
| CPC (−) | 100% (54/54) | 100% (54/54) | 91% (31/33) | 77% (14/17) |
| | | $P = 0.00006$ | $P = 0.0001$ | $P = 0.02$ |

men continued to experience biochemical failure after 5 years (Table 5 and Figure 2).

*3.2.8. Frequency of Biochemical Failure in CPC Positive and Negative Men with Time from First Blood Sample.* Men CPC positive had a higher frequency of biochemical failure at 1, 2, and 3 years of followup (Figure 2).

## 4. Discussion

The object of this study was to describe the prevalence of CPCs after radical prostatectomy. The high rate of dissemination prior to treatment has been used as a sequential method to detect prostate cancer [7]; however with surgical removal of the primary tumor, the primary source of circulating tumor cells is eradicated. Circulating tumor cells detected after primary treatment (secondary CPCs) therefore disseminate from a micrometastatic focus which may be local from the prostate bed or surrounding tumor or systemic from distant tissues. 40.2% of cases without evidence of biochemical failure had secondary CPCs detected using standard gel differential centrifugation and immunocytochemistry, including patients initially CPC negative and with the appearance of secondary CPCs >5 years after surgery. The 40.2% of men positive for secondary CPCs is less than the 57% of men with DTCs after prostatectomy reported by Morgan et al. [3]. However, our study group included stage T1 patients which may explain this difference. An alternative explanation is that CPCs are actively disseminating tumor cells; thus in patients without active dissemination of tumor cells but with dormant bone marrow micrometastasis the frequency of CPC detection will be less as has been suggested [5].

The population studied experienced biochemical failure in 32.6% of patients, comparable to the internationally published data. Known clinical-pathological risk factors correlated with the occurrence of biochemical failure were associated with a higher frequency of CPC detection, except for perineural invasion.

Not all these clinic-pathological risk factors were associated with biochemical failure; positive margins and compromise of the capsule by tumor were not associated. Perhaps more importantly the presence of secondary CPCs was associated with biochemical failure independent of these clinicopathological variables. Assuming that patients negative for CPC detection and known risk factor had the least possibility of biochemical failure, the relative risk of failure was significantly higher in patients CPC positive independent of the status of the clinical variable.

Surprisingly the presence of positive margins was not associated with biochemical failure, maybe due to the short time of followup in our patients. Ploussard et al. [8] reported that although positive margins were detected in 25.6% of cases, only 14.7% of the 1943 patients studied experience biochemical failure, the 5-year biochemical free survival being reported as 57.5% in margin positive patients compared with 84.4% in men margin negative. In men with pT2N0 cancer positive margins were not associated with biochemical failure [9].

Biochemical failure has been associated with perineural inversion [10], but there are conflicting reports [11] where a significant association has not been demonstrated. Vascular invasion has been reported to be associated with biochemical failure but added minimally to prediction models incorporating established risk factors during short follow up periods [12].

Our data using CPCs differs from the data reported by Morgan et al. [3] using the detection of DTCs in bone marrow aspirates, where the surgical margin was not associated with DTCs nor was pathological stage. There was a trend for increasing CPC detection frequency associated with increasing serum PSA and increasing Gleason score, which again was not seen in the study of DTCs by Morgan et al. [3]. However, in contrast there are published reports that primary CTCs, DTCs and micrometastasis are not associated with the Gleason score before primary therapy and that DTCs and micrometastasis after primary therapy are associated with Gleason score [5]. What may be important is that although increasing Gleason score was associated with an increased frequency of CPCs detected, in patients after radical prostatectomy those with CPCs had an increased risk of biochemical failure independent of Gleason score. This can be explained by that patients with higher Gleason scores have a higher chance of having subpopulations of cancer cells that can disseminate and implant in distant tissues. However, patients with implanted or micrometastatic cells all have a

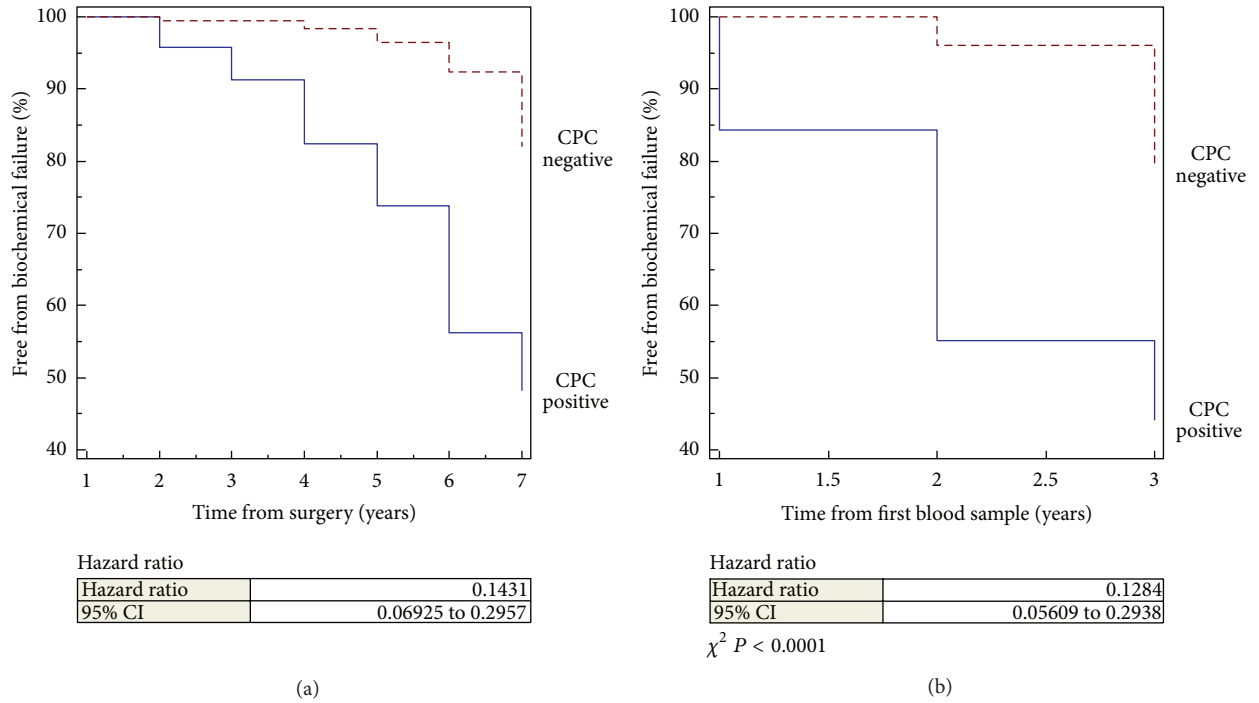



Figure 2: Kaplan-Meier plot time free of biochemical failure from time of (a) surgery and (b) blood sampling.

higher risk of biochemical failure and thus are independent of Gleason score.

Our results differ from those using DTCs, which may be explained in part by the use of differing biomarkers. Morgan et al. used anti-Ber4 anti-epithelial antibody, while we used anti-PSA. In higher grade tumors there can be decreased epithelial antigen expression and if as suggested that DTCs are circulating tumor cells, the transition epithelial mesodermal that is suggested to occur during dissemination may account for decreased epithelial antigen expression. The widely accepted concept that all cytokeratin and/or EpCAM positive, CD45 negative cells with a nucleus in cancer patients are circulating tumor cells (CTCs) has imposed a clear bias on the study of CTCs. Mainly the failure to include tumor cells that have reduced or absent cytokeratin and/or EpCAM expression and the failure to identify such cell types limit investigations into additional tumor types. EpCam is expressed in most but not all tumors [13]; there is downregulation with cancer progression and metastasis; cytokeratins are heterogeneously expressed in tumor cells and also may be downregulated during disease progression or in poorly differentiated tumors. During the progression of epithelial to mesenchymal transition both markers are downregulated [14]; EpCAM may be downregulated to allow epithelial cell dissociation from the tumor and cytokeratin downregulated to facilitate cell plasticity and migration [15]. However, Fizazi et al. [16], using anti-BerEP-4 epithelial antigen combined with telomerase activity, detected primary CPCs in 79% of patients with localized cancer, which suggests that the anti-BerEP-4 may be appropriate to detect DTCs.

To date, there are few published studies evaluating the significance of CPCs in prostate cancer patients after radical prostatectomy. Using rt-PCR in 50 patients it was reported that in men with a rising PSA 47% of patients had CPCs detected in comparison with 3% without a rising PSA [17]. In men with biochemical failure after radical prostatectomy the detection of CPCs was associated with a shorter PSA doubling time [18].

There is a clear need to identify the role of secondary CPCs in prostate cancer and also to determine on a biological level what mechanisms enable prostate cancer to recur after many years without detection. Our results indicate that a large proportion of patients with no evidence of disease have CPCs detectable after surgery, and they may reappear after a period of time of being CPC negative. These positive patients have a higher risk of biochemical failure and it suggests that tumor dormancy plays a prominent role in prostate cancer recurrence after definitive therapy. We suggest that men who become CPC positive after prostatectomy radical have dormant micrometastasis that may eventually activate and cause metastasis.

The observations from our study must be taken in the context of a population of 92 patients and although the median followup is only two years there were sufficient biochemical failures to make some general observations. Firstly CPC detection using standard immunocytochemisrty is able to identify a high risk group for biochemical failure before there is a rise in the serum PSA. By using a positive/negative result and not a defined cutoff point of a determined number of cells/mL blood it gives the treating

physician a yes/no answer. The time from surgery does not influence the interpretation of the test in men with a serum PSA <0.2 ng/mL. We sought to maximize sensitivity of the test by utilizing a single CPC cutoff. A higher cutoff would have decreased false positives, but the correlation between CPCs and biochemical recurrence presented here supports the single cell definition.

Obtaining blood samples for CPC detection is less invasive than the use of bone marrow specimens and thus could be more frequently repeated during followup.

In summary secondary CPCs are associated with increased biochemical failure in men with a PSA <0.2 ng/mL after radical prostatectomy; the presence of secondary CPCS is independent of the clinicopathological parameters normally used to predict risk of biochemical failure; however the presence of secondary CPCs does not determine if the recurrence is due to local or systemic disease. These results warrant larger studies to confirm the findings.

## Conflict of Interests

There was no conflict of Interests.

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## References

[1] C. R. Pound, A. W. Partin, M. A. Eisenberger, D. W. Chan, J. D. Pearson, and P. C. Walsh, "Natural history of progression after PSA elevation following radical prostatectomy," *Journal of the American Medical Association*, vol. 281, no. 17, pp. 1591–1597, 1999.

[2] C. L. Amling, M. L. Blute, E. J. Bergstralh, T. M. Seay, J. Slezak, and H. Zincke, "Long-term hazard of progression after radical prostatectomy for clinically localized prostate cancer: continued risk of biochemical failure after 5 years," *Journal of Urology*, vol. 164, no. 1, pp. 101–105, 2000.

[3] T. M. Morgan, P. H. Lange, M. P. Porter et al., "Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence," *Clinical Cancer Research*, vol. 15, no. 2, pp. 677–683, 2009.

[4] B. J. Bain, "Bone marrow biopsy morbidity and mortality," *British Journal of Haematology*, vol. 121, no. 6, pp. 949–951, 2003.

[5] N. P. Murray, E. Reyes, P. Tapia et al., "Redefining micrometastasis in prostate cancer-a comparison of circulating prostate cells, bone marrow disseminated tumor cells and micrometastasis: implications in determining local or systemic treatment for biochemical failure after radical prostatectomy," *International Journal of Molecular Medicine*, vol. 30, no. 4, pp. 896–904, 2012.

[6] J. G. Moreno, C. M. Croce, R. Fischer et al., "Detection of hematogenous micrometastasis in patients with prostate cancer," *Cancer Research*, vol. 52, no. 21, pp. 6110–6112, 1992.

[7] N. P. Murray, E. Reyes, P. Tapia et al., "Diagnostic performance of malignant prostatic cells detection in blood for early detection of prostate cancer: comparison to prostatic biopsy," *Archivos Españoles de Urología*, vol. 64, no. 10, pp. 961–971, 2011.

[8] G. Ploussard, M. A. Agamy, O. Alenda et al., "Impact of positive surgical margins on prostate-specific antigen failure after radical prostatectomy in adjuvant treatment-naïve patients," *British Journal of Urology International*, vol. 107, no. 11, pp. 1748–1754, 2011.

[9] R. J. Palisaar, M. Graefen, P. I. Karakiewicz et al., "Assessment of clinical and pathologic characteristics predisposing to disease recurrence following radical prostatectomy in men with pathologically organ-confined prostate cancer," *European Urology*, vol. 41, no. 2, pp. 155–161, 2002.

[10] H. G. Jeon, J. Bae, J. S. Yi, I. S. Hwang, S. E. Lee, and E. Lee, "Perineural invasion is a prognostic factor for biochemical failure after radical prostatectomy," *International Journal of Urology*, vol. 16, no. 8, pp. 682–686, 2009.

[11] A. D. Merrilees, P. B. Bethwaite, G. L. Russell, R. G. Robinson, and B. Delahunt, "Parameters of perineural invasion in radical prostatectomy specimens lack prognostic significance," *Modern Pathology*, vol. 21, no. 9, pp. 1095–1100, 2008.

[12] D. S. Yee, S. F. Shariat, W. T. Lowrance et al., "Prognostic significance of lymphovascular invasion in radical prostatectomy specimens," *British Journal of Urology International*, vol. 108, no. 4, pp. 502–507, 2011.

[13] P. T. Went, A. Lugli, S. Meier et al., "Frequent EpCam protein expression in human carcinomas," *Human Pathology*, vol. 35, no. 1, pp. 122–128, 2004.

[14] P. Paterlini-Brechot and N. L. Benali, "Circulating tumor cells (CTC) detection: clinical impact and future directions," *Cancer Letters*, vol. 253, no. 2, pp. 180–204, 2007.

[15] C. Raimondi, A. Gradilone, G. Naso et al., "Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients," *Breast Cancer Research and Treatment*, vol. 130, no. 2, pp. 449–455, 2011.

[16] K. Fizazi, L. Morat, L. Chauveinc et al., "High detection rate of circulating tumor cells in blood of patients with prostate cancer using telomerase activity," *Annals of Oncology*, vol. 18, no. 3, pp. 518–521, 2007.

[17] R. Millon, D. Jacqmin, D. Muller, J. Guillot, M. Eber, and J. Abecassis, "Detection of prostate-specific antigen- or prostate-specific membrane antigen-positive circulating cells in prostatic cancer patients: clinical implications," *European Urology*, vol. 36, no. 4, pp. 278–285, 1999.

[18] B. Tombal, P. J. Van Cangh, S. Loric, and J. L. Gala, "Prognostic value of circulating prostate cells in patients with a rising PSA after radical prostatectomy," *Prostate*, vol. 56, no. 3, pp. 163–170, 2003.

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*Clinical Study*

# Are Preoperative Kattan and Stephenson Nomograms Predicting Biochemical Recurrence after Radical Prostatectomy Applicable in the Chinese Population?

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*Purpose*. Kattan and Stephenson nomograms are based on the outcomes of patients with prostate cancer recruited in the USA, but their applicability to Chinese patients is yet to be validated. We aim at studying the predictive accuracy of these nomograms in the Chinese population. *Patients and Methods*. A total of 408 patients who underwent laparoscopic or open radical resection of prostate from 1995 to 2009 were recruited. The preoperative clinical parameters of these patients were collected, and they were followed up regularly with PSA monitored. Biochemical recurrence was defined as two or more consecutive PSA levels >0.4 ng/mL after radical resection of prostate or secondary cancer treatment. *Results*. The overall observed 5-year and 10-year biochemical recurrence-free survival rates were 68.3% and 59.8%, which was similar to the predicted values by the Kattan and Stephenson nomograms, respectively. The results of our study achieved a good concordance with both nomograms (Kattan: 5-years, 0.64; Stephenson: 5-years, 0.62, 10-years, 0.71). *Conclusions*. The incidence of prostate cancer in Hong Kong is increasing together with the patients' awareness of this disease. Despite the fact that Kattan nomograms were derived from the western population, it has been validated in our study to be useful in Chinese patients as well.

## 1. Introduction

Patients' awareness on prostate cancer is increasing together with its incidence in Hong Kong [1]. According to the latest publication by the Hong Kong Cancer Registry in 2008, prostate cancer is the third most common and the fifth leading cause of cancer deaths in Hong Kong males [1]. An objective tool, such as the Kattan and Stephenson nomograms [2, 3], to estimate the expected outcome would be essential for surgeons in decision making and in counseling the patients on prognosis after treatment. Kattan nomograms were first developed in 1998 [2] and later enhanced by Stephenson et al. in 2006 [3], based on the outcome results of patients with prostate cancer recruited in the USA. It has been validated by patient populations from the United States, Australia, and Europe [4–8], but its applicability to Chinese patients is yet to be validated. We aim at studying the predictive accuracy of Kattan nomograms in the Chinese population based on our center's results.

## 2. Patient and Methods

From 1995 to 2009, 408 patients who underwent laparoscopic or open radical prostatectomy for prostate carcinoma were recruited. The clinical stage of prostate cancer (according to 2009 TNM classification system), the pre-op PSA level, the number of positive and negative cores by transrectal ultrasound guided prostate biopsy, and Gleason Scores of the specimens were collected for analysis. All patients were followed up regularly with PSA monitored, and biochemical recurrence was defined as two or more consecutive PSA level greater than 0.4 ng/mL after radical prostatectomy or secondary cancer treatment [2]. 82 patients who had missing data (such as PSA and Biopsy results) or defaulted followup were excluded from the study. The observed biochemical recurrence rates, calculated by the Kaplan-Meier analysis, were compared with the values predicted by the Kattan nomograms. In order to facilitate the calculation of the concordance index (Range: 0.5 to 1) [9–12], the population was

Table 1: Background demographics of patients with prostate cancer undergoing prostatectomy.

| Variables | Kattan 1998 | Stephenson 2006 | Our study |
|---|---|---|---|
| PSA | $N = 983$ | $N = 1978$ | $N = 326$ |
| <4 | 217 (22.1%) | Median PSA = 6.1 (Range: 4.4–9.0) | 28 (8.6%) |
| 4.1–10 | 472 (48%) | | 136 (41.7%) |
| 10.1–20 | 187 (19%) | | 102 (31.3%) |
| >20 | 107 (10.9%) | | 60 (18.4%) |
| Clinical stage | | | |
| T1a/b | 83 (8.4%) | 0 (0%) | 33 (10.1%) |
| T1c | 148 (15.1%) | 803 (40.6%) | 224 (68.7%) |
| T2a | 266 (27.1%) | 509 (25.7%) | 32 (9.8%) |
| T2b | 246 (25.0%) | 335 (17.0%) | 21 (6.5%) |
| T2c | 182 (18.5%) | 244 (12.3%) | 10 (3.1%) |
| T3 | 58 (5.9%) | 88 (4.4%) | 6 (1.8%) |
| Gleason score (GS) | | | |
| GS 1-2/1-2 | 108 (11%) | | 0 (0.0%) |
| GS 1-2/3 | 158 (16.1%) | | 1 (0.3%) |
| GS 3/3 and 3/1-2 | 405 (41.2%) | | 153 (46.9%) |
| GS 3/4-5 | 213 (21.7%) | | 103 (31.6%) |
| GS 4-5/1-5 | 99 (10.1%) | | 69 (21.2%) |
| GS 2–6 | | 1348 (68%) | 154 (47.2%) |
| GS 3+4 | | 397 (20%) | 98 (30.1%) |
| GS 4+3 | | 130 (7%) | 41 (12.6%) |
| GS 8–10 | | 104 (5%) | 33 (10.1%) |

divided into 5 groups (based on similar Kattan scores) with almost equal number of patients in each group. Concordance index can be defined as the proportion of randomly paired patients for whom the patient with the greater probability of recurrence also had earlier disease recurrence [2, 4, 6, 9–12]. SPSS version 19 was used for all statistical analyses.

## 3. Results

The baseline demographics of the patients in our cohort were shown to have higher PSA values and Gleason scores as compared to the Kattan cohorts (Table 1). Thus, the overall observed 5-year (68.3%) and 10-year (59.8%) biochemical recurrence free survival rates were slightly lower than those of Kattan's cohort (Figures 1 and 2). However, these two observed values were similar to the predicted values derived from the 1998 Kattan and 2006 Stephenson nomograms. The plots comparing the observed and predicted 5-year and 10-year biochemical recurrence rates demonstrated that our study results achieved a good concordance with both Kattan nomograms (Figures 3, 4, and 5). For the 1998 version, the 5-year concordance rate was 0.64, whereas for the 2006 version, the 5-year and 10-year values were 0.62 and 0.71, respectively.

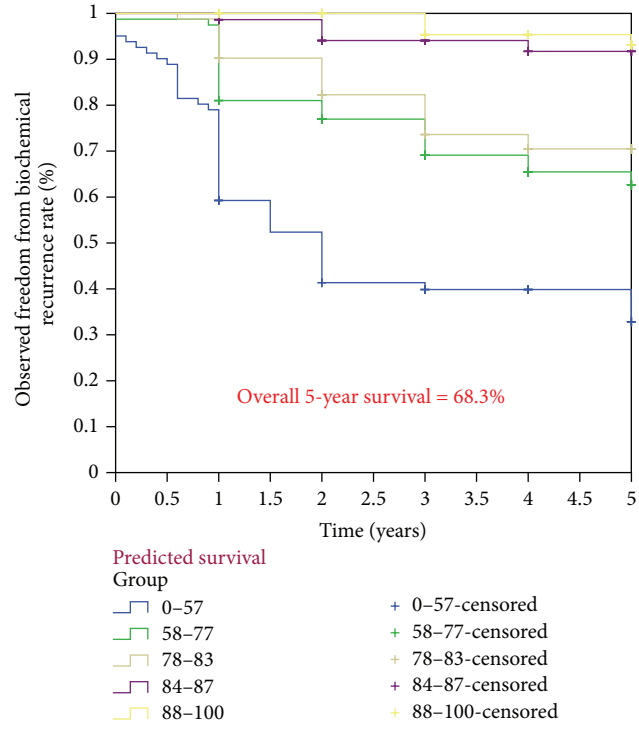


Figure 1: Observed freedom from biochemical recurrence rates according to the 1998 Kattan nomogram.

## 4. Discussion

The incidence of prostate cancer is increasing in Hong Kong with 1369 new cases diagnosed in 2008 [1]. An objective tool to estimate the expected outcome would be essential for surgeons in decision making and counselling the patients on prognosis after surgical treatment. There are multiple nomograms generated for the calculation of post-prostatectomy outcomes [13–18]. Kattan nomogram is the one of the most accepted guidelines in determining the biochemical recurrence-free survival rate after prostatectomy. It was generated based on a cohort of patients with prostate cancer recruited in the USA, and had been validated in various studies [4–8]. However, there is a possibility that the Kattan nomograms might not be applicable to Chinese population, because of the different risks of prostate cancer between Asian and Western populations [19–25]. Thus, we perform the validation of Kattan nomograms in Chinese patients with our data collected in Hong Kong.

We have inferior patient background demographics as compared to the Kattan cohort, and thus, our 5-year and 10-year biochemical recurrence rates were higher. However, our data demonstrated a good concordance index with the expected results. The overall observed 5-year and 10-year biochemical recurrence free survival rates were similar to the predicted values by the 1998 Kattan and 2006 Stephenson cohorts respectively. The concordance indexes of our study (5 year, 1998 version: 0.64, 5-year and 10-year, 2006 version: 0.62 and 0.71) were similar to those of Kattan's external validation cohort [2] (5-year: 0.64) as well as Stephenson's cohort [3]

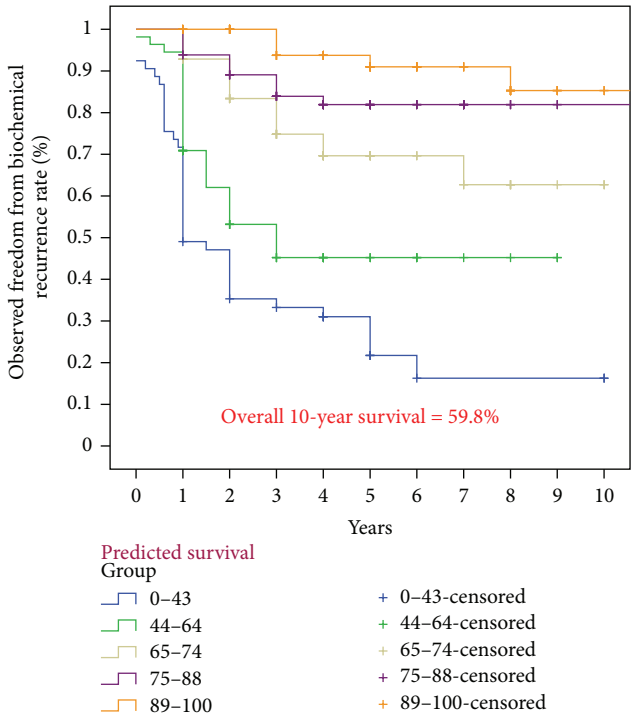


Figure 2: Observed freedom from biochemical recurrence rates according to the 2006 Stephenson nomogram.

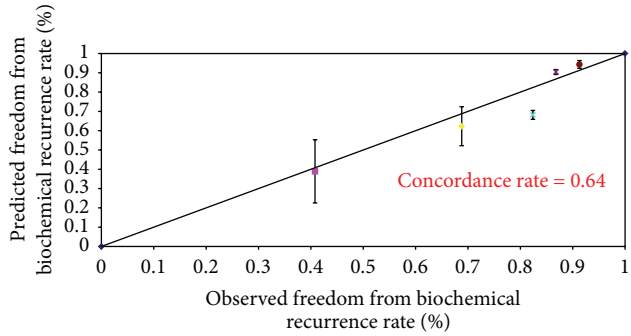


Figure 3: Observed versus predicted 5-year freedom from biochemical recurrence rates according to the 1998 Kattan nomogram.

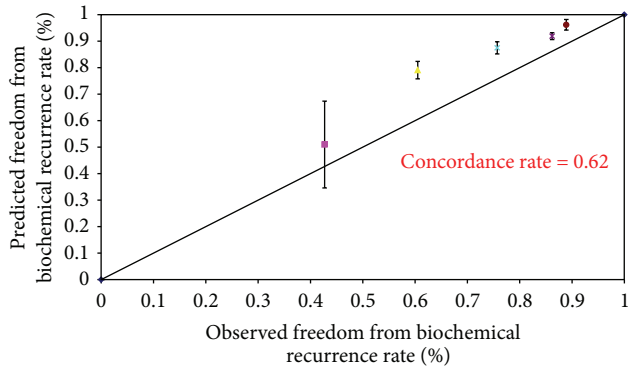


Figure 4: Observed versus predicted 5-year freedom from biochemical recurrence rates according to the 2006 Stephenson nomogram.

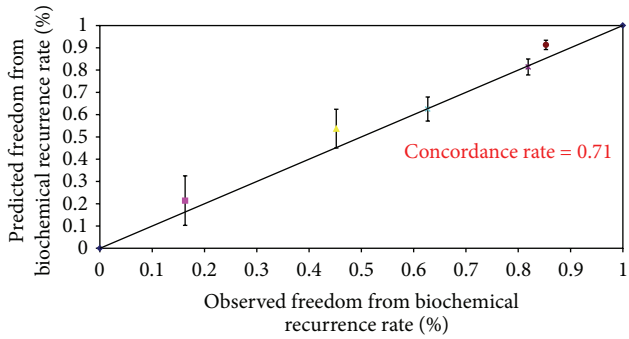


Figure 5: Observed versus predicted 10-year freedom from biochemical recurrence rates according to the 2006 Stephenson nomogram.

(5-year: 0.6, 10-years: 0.71). Other studies (Eskicorapci et al. [6]—5-year, 1998 version: 0.698, 10-year, 2006 version: 0.705 and Jr. et al. [5]—5-year: 0.74) in Turkish and African men also showed comparable concordance indexes as to our study. This suggested that the Kattan nomograms could be applied to different populations over the world, including the Chinese population as supported by our data. Despite the fact that the Kattan nomograms showed a good concordance with our cohort results, the 2006 version slightly overestimated the 5-year biochemical recurrence free survival rates.

There were several limitations in our study. First, our data was based on a single institution, though the operations were performed by various surgeons. Second, our cohort population size was relatively small, and a larger sample size may demonstrate a better concordance with the Kattan nomograms.

## 5. Conclusion

Incidence of prostate cancer in Hong Kong is increasing together with patients' awareness on this disease. Despite the fact that Kattan nomograms were derived from the western population, it has been validated in our study to be useful in Chinese patients as well. The 1998 Kattan nomogram can slightly better predict the outcome of the 5-year biochemical recurrence free survival than the 2006 version. The 2006 version achieves a satisfactory concordance with the 10-year outcome of our cohort. In view of the promising results, Kattan nomograms have been implemented into our clinical practice in managing patients with prostate cancer.

## References

[1] *Fact Stats for Prostate Cancer*, Hong Kong Cancer Registry, Hospital Authority, 2008.

[2] M. W. Kattan, J. A. Eastham, A. M. F. Stapleton, T. M. Wheeler, and P. T. Scardino, "A preoperative nomogram for disease recurrence following radical prostatectomy for prostate cancer," *Journal of the National Cancer Institute*, vol. 90, no. 10, pp. 766–771, 1998.

[3] A. J. Stephenson, P. T. Scardino, J. A. Eastham et al., "Preoperative nomogram predicting the 10-year probability of prostate

cancer recurrence after radical prostatectomy," *Journal of the National Cancer Institute*, vol. 98, no. 10, pp. 715–717, 2006.

[4] M. Graefen, P. I. Karakiewicz, I. Cagiannos et al., "International validation of a preoperative nomogram for prostate cancer recurrence after radical prostatectomy," *Journal of Clinical Oncology*, vol. 20, no. 15, pp. 3206–3212, 2002.

[5] F. J. Bianco Jr., M. W. Kattan, P. T. Scardino, I. J. Powell, J. E. Pontes, and D. P. Wood, "Radical prostatectomy nomograms in black American men: accuracy and applicability," *Journal of Urology*, vol. 170, no. 1, pp. 73–76, 2003.

[6] S. Y. Eskicorapci, L. Türkeri, E. Karabulut et al., "Validation of two preoperative kattan nomograms predicting recurrence after radical prostatectomy for localized prostate cancer in Turkey: a multicenter study of the uro-oncology society," *Urology*, vol. 74, no. 6, pp. 1289–1295, 2009.

[7] K. L. Greene, M. V. Meng, E. P. Elkin et al., "Validation of the Kattan preoperative nomogram for prostate cancer recurrence using a community based cohort: results from cancer of the prostate strategic urological research endeavor (CaPSURE)," *Journal of Urology*, vol. 171, no. 6, part 1, pp. 2255–2259, 2004.

[8] H. Isbarn, P. I. Karakiewicz, J. Walz et al., "External validation of a preoperative nomogram for prediction of the risk of recurrence after radical prostatectomy," *International Journal of Radiation Oncology Biology Physics*, vol. 77, no. 3, pp. 788–792, 2010.

[9] M. G. Kendall and B. Babington Smith, "The problem of m rankings," *The Annals of Mathematical Statistics*, vol. 10, no. 3, pp. 275–287, 1939.

[10] G. W. Corder and D. I. Foreman, *Nonparametric Statistics for Non-Statisticians: A Step-By-Step Approach*, Wiley, 2009.

[11] Y. Dodge, *The Oxford Dictionary of Statistical Terms*, OUP, 2003.

[12] P. Legendre, "Species associations: the Kendall coefficient of concordance revisited," *Journal of Agricultural, Biological, and Environmental Statistics*, vol. 10, no. 2, pp. 226–245, 2005.

[13] B. A. O'Brien, R. J. Cohen, T. M. Wheeler, and R. E. Moorin, "A post-radical-prostatectomy nomogram incorporating new pathological variables and interaction terms for improved prognosis," *Journal of the British Association of Urological Surgeons*, vol. 107, no. 3, pp. 389–395, 2011.

[14] C. R. Porter, N. Suardi, U. Capitanio et al., "A nomogram predicting prostate cancer-specific mortality after radical prostatectomy," *Urologia Internationalis*, vol. 84, no. 2, pp. 132–140, 2010.

[15] J. S. Chung, H. Y. Choi, H. R. Song et al., "Preoperative nomograms for predicting extracapsular extension in korean men with localized prostate cancer: a multi-institutional clinicopathologic study," *Journal of Korean Medical Science*, vol. 25, no. 10, pp. 1443–1448, 2010.

[16] J. Walz, F. K. H. Chun, E. A. Klein et al., "Nomogram predicting the probability of early recurrence after radical prostatectomy for prostate cancer," *Journal of Urology*, vol. 181, no. 2, pp. 601–608, 2009.

[17] C. R. Porter, N. Suardi, K. Kodama et al., "A nomogram predicting metastatic progression after radical prostatectomy," *International Journal of Urology*, vol. 15, no. 10, pp. 889–894, 2008.

[18] S. Naito, K. Kuroiwa, N. Kinukawa et al., "Validation of Partin tables and development of a preoperative nomogram for Japanese patients with clinically localized prostate cancer using 2005 International Society of Urological Pathology consensus on Gleason grading: data from the Clinicopathological Research Group for Localized Prostate Cancer," *Journal of Urology*, vol. 180, no. 3, pp. 904–910, 2008.

[19] K. Kawamura, H. Suzuki, N. Kamiya et al., "Development of a new nomogram for predicting the probability of a positive initial prostate biopsy in Japanese patients with serum PSA levels less than 10 ng/mL," *International Journal of Urology*, vol. 15, no. 7, pp. 598–603, 2008.

[20] E. M. Raymundo, K. R. Rice, Y. Chen, J. Zhao, and S. A. Brassell, "Prostate cancer in Asian Americans: incidence, management and outcomes in an equal access healthcare system," *Journal of the British Association of Urological Surgeons*, vol. 107, no. 8, pp. 1216–1222, 2011.

[21] L. S. Cook, M. Goldoft, S. M. Schwartz, and N. S. Weiss, "Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants," *Journal of Urology*, vol. 161, no. 1, pp. 152–155, 1999.

[22] S. S. Lin, C. A. Clarke, A. W. Prehn, S. L. Glaser, D. W. West, and C. D. O'Malley, "Survival differences among Asian subpopulations in the United States after prostate, colorectal, breast, and cervical carcinomas," *Cancer*, vol. 94, no. 4, pp. 1175–1182, 2002.

[23] M. McCracken, M. Olsen, M. S. Chen et al., "Cancer incidence, mortality, and associated risk factors among Asian Americans of Chinese, Filipino, Vietnamese, Korean, and Japanese ethnicities," *CA Cancer Journal for Clinicians*, vol. 57, no. 4, pp. 190–205, 2007.

[24] A. Man, T. Pickles, K. N. Chi et al., "Asian race and impact on outcomes after radical radiotherapy for localized prostate cancer," *Journal of Urology*, vol. 170, no. 3, pp. 901–904, 2003.

[25] A. S. Robbins, T. M. Koppie, S. L. Gomez, A. Parikh-Patel, and P. K. Mills, "Differences in prognostic factors and survival among white and Asian men with prostate cancer, California, 1995–2004," *Cancer*, vol. 110, no. 6, pp. 1255–1263, 2007.

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*Clinical Study*

# Laparoscopic Radical Prostatectomy: The Learning Curve of a Low Volume Surgeon

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*Objective*. Analyze the learning curve for laparoscopic radical prostatectomy in a low volume program. *Materials and Methods*. A single surgeon operated on 165 patients. Patients were consecutively divided in 3 groups of 55 patients (groups A, B, and C). An enhancement of estimated blood loss, surgery length, and presence of a positive surgical margin were all considered as a function of surgeon's experience. *Results*. Operative time was 267 minutes for group A, 230 minutes for group B, and 159 minutes for group C, and the operative time decreased over time, but a significant difference was present only between groups A and C ($P < 0.001$). Mean estimated blood loss was 328 mL, 254 mL, and 206 mL ($P = 0.24$). A conversion to open surgery was necessary in 4 patients in group A. Positive surgical margin rates were 29.1%, 21.8%, and 5.5% ($P = 0.02$). Eight patients in group A, 4 patients in group B, and one in group C had biochemical recurrence. *Conclusion*. Significantly less intraoperative complications were evident after the first 51 cases. All other parameters (blood loss, operative time, and positive surgical margins) significantly decreased and stabilized after 110 cases. Those outcomes were somehow similar to previous published series by high-volume centers.

## 1. Introduction

Introduction of the laparoscopic approach has revolutionized the field of minimal invasive surgery, and in the modern era laparoscopic radical prostatectomy (LRP) has been described as a standard and reproducible surgical procedure in many centers worldwide [1].

In the same way of other modern surgical techniques, laparoscopic procedures necessitate new training methods and an amount of surgical procedures performed before a surgeon reaches an accepted stage of expertise in outcome parameters. The length of this learning curve is proportional to the complexity of the procedure. Several authors have published their results, and some learning curves, about laparoscopic radical prostatectomy, considered a highly complex surgery [2–4].

However, most of these studies have been conducted in large academic centers with a high volume of radical prostatectomies. Whether those same learning curves are reproducible in low volume centers by less experienced surgeons remains an unanswered question. Therefore, in this paper, we aimed to analyze the learning curve for extraperitoneal laparoscopic radical prostatectomy in a low volume environment.

## 2. Materials and Methods

Between August 2003 and June 2011, we have performed 165 LRP procedures. The study had been authorized by the appropriate ethics committee, and informed consents obtained. Patients were ordered chronologically from number 1 to number 165 for this study. The patients were divided into 3 groups for prospective analysis: group 1 consisted of the first 55 patients, group 2 was formed by the patients numbered from 56 to 110, and the final 55 patients were included in the group 3.

All surgeries were performed by a single surgeon (AIM), and all patients were diagnosed previously as localized prostatic adenocarcinoma. All procedures were performed using the extraperitoneal surgical technique using five trocars

[5]. Antegrade dissection was done, and the prostate pedicles controlled with polymer clips (Hem-o-lock, Weck Closure Systems, Research Triangle. Park NC, USA). Urethrovesical anastomosis was performed with a running suture, and lymphadenectomy performed in the presence of a PSA higher than 20 ng/dL, Gleason 7 (4 + 3) or higher, or presence of suspicious nodes described in preoperative imaging studies.

Patients were then treated following the local hospital clinical pathway of LRP, and postoperative analgesia included administration of nonsteroidal anti-inflammatory drugs intravenously during the first 24 h and orally thereafter until discharge. On the first postoperative day physical therapists assisted in mobilization, and patients were reefed the evening of the day of surgery. All radical prostatectomy specimens were evaluated by a specialized uropathologist.

The length of surgery, intraoperative blood loss, transfusion rates, intra- and postoperative complications, conversion, histopathologic results, and oncological outcome (PSA recurrence) were determined in all groups and compared. All patients were discharged with a temporary bladder catheter in place, which was typically removed on the seventh day after surgery. Complications were classified according to the 2004 Clavien-Dindo classification [6].

Postoperative erectile dysfunction and incontinence were not evaluated in this paper. A positive surgical margin was defined as the presence of tumour cells in contact with the inked surface of the specimen.

In order to establish the learning curve, all variables were calculated and recorded, and a comparison between the groups was performed. Statistical analysis was performed with the SPSS program (Statistical Package for Social Sciences, version 11.01, Chicago, IL). For qualitative variables the absolute ($n$) and relative (%) frequencies were recorded. The chi-square analysis was used to compare those variables among them. Significance was established at $P < 0.05$.

ANOVA was used to compare variables with continuous values. In order to evaluate the equality of group variances the Brown-Forsythe test was also applied. In the presence of significant differences between the groups, comparisons were then performed using the Bonferroni test.

## 3. Results

For the 165 patients, we observed a median age of 61.7 (44–83) years, a median prostate size of 39.07 g (15–150 g), and a median preoperative PSA of 6.66 ng/mL (1.8–39.9); those values were equivalent in all groups ($P > 0.05$).

The parameters found in groups A, B, and C, respectively, were the following: mean surgical time: 267.1 (±64.3), 230 (±65.0), and 159.5 (±35.5) min ($P < 0.001$). Loss of blood was 328 (±188), 254 (±129), and 206 (±95) mL, and a significant difference was present only between groups A and C ($P < 0.001$). Eleven patients (20.0%) in group A and one (1.8%) in group B required blood transfusion. No transfusions were necessary in group C ($P = 0.010$).

Prolonged urine leakage was observed in seven patients (12.7%) in group A and in seven patients (12.7%) in group B.

Rectal lesions occurred in 3 patients (5.4%) in group A; the injuries were repaired intraoperatively with polyglactin 3–0 sutures in two planes. Another patient in group A sustained an intraoperative bladder injury and also repaired laparoscopically. No intraoperative lesions were recorded in groups B or C.

An analysis of postoperative complications (Clavien-Dindo classification) showed a significant difference between the groups ($P < 0.05$). Clavien I and III were more frequent in group B and II in group A. Two patients in group C presented postoperative issues (Table 1).

Conversion to open surgery was deemed necessary in 4 patients (7.3%) in group A. No other conversions were then required.

In groups A, B and C, margins were respectively positive on: 29.1% (16), 21.8% (12) and 5.5% (3) patients. When positive margins were correlated to the clinical stage of the disease a higher incidence was demonstrated in pT3a and pT3b individuals when compared to other patients (Table 2).

Due to a high risk of local relapse, 13.9% (23) of patients received radiotherapy.

After a minimum followup of 20 months (20–97 months), 8 patients in group A, 4 patients in group B, and one patient in group C had biochemical recurrence (PSA < 0.2 ng/mL).

## 4. Discussion

Historically, open radical prostatectomy is the standard surgical treatment for localized prostate cancer in patients in good health [10]. However, this procedure was not widely accepted until 1982 when a refined and reproducible method was described by Walsh and Donker [11]. Sixteen years later a laparoscopic technique for the management of localized prostate cancer was suggested by Schuessler et al, but the conclusions learned from the initial series were that this was a lengthy and difficult procedure, and little advantage was added compared to the open counterpart [12].

The initial procedure was revised [13] and over the latest years, the laparoscopic technique has shown substantial efficacy [4]. The benefits of the minimally invasive approach were reported in several series, but until recently, the procedure was limited to specialized centers, mainly due to a steep learning curve.

This difficulty is attributed to the counter-intuitive motion, two-dimensional visualization, and lack of articulating instruments for standard laparoscopic surgery. Several authors have evaluated their initial series, and some learning curves were proposed, mainly in academic centers with high surgery volume [8, 14, 15].

On the other hand, even after reviewing large series it seems it is not yet possible to estimate the number of cases required for a novice surgeon to master the skills necessary to perform a laparoscopic radical prostatectomy. In a review of their first 1311 cases Vallancien et al. [16] suggested that at least 50 difficult operations, with at least one case/week during the first year, were required to master complex laparoscopic urological procedures. Conversely, the records from 8,544 consecutive patients with prostate cancer treated laparoscopically by 51 surgeons at 14 academic institutions in Europe and the USA were evaluated in a multicenter study evaluating the presence of positive surgical margins

Table 1: Postoperative complications (Clavien-Dindo classification).

| Complication/Clavien | A | | B | | C | | *P* value |
|---|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % | |
| Urinary extravasation, temporary elevated creatinine/I | 8 | 14.5% | 8 | 14.5% | 1 | 1.8% | 0.001 |
| Blood transfusion/II | 11 | 20% | 1 | 1.8% | 0 | 0% | |
| Hem-o-lock clip migration to bladder (cystoscopy under local anesthesia)/IIIa | 0 | 0% | 0 | 0% | 1 | 1.8% | |
| Management of urinoma or infected lymphocele, embolization and colostomy (intervention under general anesthesia)/IIIb | 3 | 5.4% | 4 | 7.3% | 0 | 0% | |
| Acute pulmonary edema/IV | 1 | 1.8% | 0 | 0.0 % | 0 | 0% | |

Table 2: Positive margins versus stage of the disease.

| TNM | Group A | | Group B | | Group C | | total | |
|---|---|---|---|---|---|---|---|---|
| | M+/total | % | M+/Total | % | M+/Total | % | M+ | % |
| pT1 | 0/2 | 0 | 0/2 | 0 | 0/2 | 0 | 0 | 0 |
| pT2a | 0/6 | 0 | 1/10 | 10.0 | 0/16 | 0 | 1 | 3.8 |
| pT2b | 0/4 | 0 | 0/1 | 0 | 1/7 | 14.3 | 1 | 12.5 |
| pT2c | 4/24 | 16.7 | 9/35 | 25.7 | 1/23 | 4.3 | 14 | 17.1 |
| pT3a | 9/14 | 64.3 | 2/5 | 40.0 | 1/5 | 20.0 | 12 | 50.0 |
| pT3b | 3/4 | 75.0 | 0/2 | 0 | 0/1 | 0 | 3 | 42.8 |
| pT3c | 0/1 | 0 | 0/0 | 0 | 0/0 | 0 | 0 | 0 |
| pT4 | 0/0 | 0 | 0/0 | 0 | 0/1 | 0 | 0 | 0 |
| Total | 16/55 | 29.1 | 12/55 | 21.8 | 3/55 | 5.5 | 31 | 18.8 |

M+: positive margin.

as an effect of the surgeons' experience, and an apparent improvement in surgical margin rates up to a plateau was demonstrated only after 200 to 250 surgeries [16].

As seen in previous reports, a drop in the complications rate was demonstrated as the surgeon's experience increased. In our experience significantly less intraoperative complications and conversions to open surgery were evident after the first 51 cases, and blood loss and operative time continued to significantly decrease and stabilized after 110 cases. On a study published by Starling et al. [17] an improvement of the surgical and functional parameters occurred after 70 cases. On the other hand, in series of high-volume academic centers residents without prior experience required 38–52 cases to be considered competent [7, 18].

This same evolution was present when oncological parameters were evaluated. In our series the positive surgical margins also significantly decrease and stabilized after 110 cases. Our overall incidence of positive surgical margins was 20%, and this was similar to the series presented by Katz et al. [19]. As expected, the incidence of positive margins in our series increased in pT3 tumors and varies in pT2 tumors among the series; this was also present when other authors presented their learning curves [19]. In a study published by Bollens et al. 11 of 50 patients presented positive surgical margins after laparoscopic prostatectomy, and of these 11 cases two were pT2 tumors [20]. Rassweiler et al. reported, after reviewing 180 patients, a 16% incidence of positive, and almost half of patients had pT3 tumors [21]. Guillonneau and Vallancien presented a 14% incidence of positive margins in pT2b tumors and 33% in pT3a [22].

In our study no patient had biochemical recurrence after 110 cases. In a study published by Vickers et al. the probability of recurrence initially dropped steeply then reached a plateau after 250–350 surgeries [23]. Similarly, a large multicenter study demonstrated a plateau also at 200 to 250 surgeries [9].

Interestingly, this same retrospective study conducted by Vickers et al. suggested that 750 laparoscopic radical prostatectomies were necessary in order to reach results equivalent to the open approach [23].

Our series was performed by a single surgeon with previous laparoscopic experience with other kinds of surgery but no direct supervision during the learning curve. Other authors suggested that the learning curve could have been shortened if a training program under supervision has been used. One proposed method is the Leipzig model, where an expert mentor acts as a first assistant, while the student performs the steps corresponding to his or her level, then the student remain as a first assistant for the remainder of the surgery, leading to a gradual learning process [24].

The selection between an extraperitoneal and a transperitoneal approaches to the laparoscopic radical prostatectomy depends nowadays mainly on the surgeon's preference. Initially, a transperitoneal approach has been elected as the main access for this procedure, along with an antegrade technique [13], which was reproduced by other centers [22]. Later, Rassweiler et al. described the feasibility of LRP done through

the Retzius space, performing the surgery in an retrograde manner and accessing the seminal vesicles after transecting the posterior bladder neck (Heilbronn technique) [21]. An extraperitoneal approach was first described by Raboy et al. [25], following the principles of the French technique and transecting the venous complex and urethra as the final portion of the prostate dissection.

In our series an extraperitoneal antegrade laparoscopic technique, performed in a similar manner as previously described by Dubernard et al. [5], was used, aiming to unite the advantages of minimally invasive techniques with those of extraperitoneal cavity surgery, also avoiding the extreme Trendelenburg position used in the transperitoneal technique, since the peritoneum retracts the intestines, allowing a more neutral position. Furthermore, this approach may also be suitable for patients with multiple previous abdominal surgeries and in the presence of obesity [26].

Our study has several limitations. Initially, only the total operative time was recorded, instead of timing separately each step of the procedure, as done by Dev et al. [3], what could have added substantial information regarding the difficulty and complexity of each step to surpass the initial learning curve. Furthermore, although we have added the biochemical recurrence as one of the parameters, we are aware that our relative short followup may limit the usage of this information in our study. Finally, continence and erectile dysfunctions were not evaluated in our series due to a lack of standardization for urinary incontinence and for the no application of validated questionnaires for erectile dysfunction, but we are aware that those parameters would add important information regarding the functional outcomes for the studied population.

Finally, it must be noted that in the modern era a laparoscopic robotic approach has been favored over pure laparoscopic surgeries mainly because of its apparent reduced learning curve [27, 28], but, especially in developing countries, its high costs, availability, and the training facilities required are still major issues that are somehow difficult to surmount, particularly in limited budget situations. Therefore, apparently, there is still a role for laparoscopic radical prostatectomy in our days, and studying the learning process and identifying a proper learning curve for this procedure in low-volume centers seem necessary even in the robotics epoch. Additionally, the surgeon with experience in laparoscopic radical prostatectomy may continue by minimally invasive surgery in cases of breakdown of the robot during the surgery avoiding conversion to open procedure and may facilitate the beginning of robotic assisted laparoscopic radical prostatectomy.

## 5. Conclusion

Although further studies seem necessary to unify and identify the number of cases required to master this technique considerable less intraoperative complications and conversions to open surgery were noted after the first 51 cases, and all other parameters (blood loss, operative time, and positive surgical margins) significantly decreased and stabilized after 110 cases in our study. The learning curve for extraperitoneal laparoscopic radical prostatectomy appears to be continuous and the implementation of a successful program was possible even in the presence of a low volume and in the absence of a specific mentorship program in the early learning curve.

## References

[1] G. S. Sandhu, K. G. Nepple, Y. S. Tanagho, and G. L. Andriole, "Laparoscopic prostatectomy for prostate cancer: continued role in urology," *Surgical Oncology Clinics of North America*, vol. 22, no. 1, pp. 125–141, 2013.

[2] S. L. Chang and M. L. Gonzalgo, "Surgery: laparoscopic prostatectomy: learning curve and cancer control," *Nature Reviews Urology*, vol. 6, no. 7, pp. 361–362, 2009.

[3] H. Dev, N. L. Sharma, S. N. Dawson et al., "Detailed analysis of operating time learning curves in robotic prostatectomy by a novice surgeon," *BJU International*, vol. 109, no. 7, pp. 1074–1080, 2012.

[4] M. Hruza, H. O. Weiß, G. Pini et al., "Complications in 2200 consecutive laparoscopic radical prostatectomies: standardised evaluation and analysis of learning curves," *European Urology*, vol. 58, no. 5, pp. 733–741, 2010.

[5] P. Dubernard, S. Benchetrit, P. Chaffange, T. Hamza, P. Van Box Som, and A. Hoznek, "Retrograde extraperitoneal laparoscopic prostatectomy (R.E.L.P.) simplified technique (based on a series of 143 cases)," *Progres en Urologie*, vol. 13, no. 1, pp. 163–174, 2003.

[6] D. Dindo, N. Demartines, and P. A. Clavien, "Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey," *Annals of Surgery*, vol. 240, no. 2, pp. 205–213, 2004.

[7] J. U. Stolzenburg, R. Rabenalt, M. Do, L. C. Horn, and E. N. Liatsikos, "Modular training for residents with no prior experience with open pelvic surgery in endoscopic extraperitoneal radical prostatectomy," *European Urology*, vol. 49, no. 3, pp. 491–498, 2006.

[8] J. Rassweiler, M. Schulze, D. Teber et al., "Laparoscopic radical prostatectomy with the heilbronn technique: oncological results in the first 500 patients," *Journal of Urology*, vol. 173, no. 3, pp. 761–764, 2005.

[9] F. P. Secin, C. Savage, C. Abbou et al., "The learning curve for laparoscopic radical prostatectomy: an international multicenter study," *Journal of Urology*, vol. 184, no. 6, pp. 2291–2296, 2010.

[10] W. J. Catalona, C. G. Ramos, and G. F. Carvalhal, "Contemporary results of anatomic radical prostatectomy," *CA: A Cancer Journal for Clinicians*, vol. 49, no. 5, pp. 282–296, 1999.

[11] P. C. Walsh and P. J. Donker, "Impotence following radical prostatectomy: insight into etiology and prevention," *Journal of Urology*, vol. 128, no. 3, pp. 492–497, 1982.

[12] W. W. Schuessler, P. G. Schulam, R. V. Clayman, and L. R. Kavoussi, "Laparoscopic radical prostatectomy: initial short-term experience," *Urology*, vol. 50, no. 6, pp. 854–857, 1997.

[13] B. Guillonneau, X. Cathelineau, E. Barret, F. Rozet, and G. Vallancien, "Laparoscopic radical prostatectomy: technical and early oncological assessment of 40 operations," *European Urology*, vol. 36, no. 1, pp. 14–20, 1999.

[14] E. J. Trabulsi and B. Guillonneau, "Laparoscopic radical prostatectomy," *Journal of Urology*, vol. 173, no. 4, pp. 1072–1079, 2005.

[15] R. Bollens, S. Sandhu, T. Roumeguere, T. Quackels, and C. Schulman, "Laparoscopic radical prostatectomy: the learning curve," *Current Opinion in Urology*, vol. 15, no. 2, pp. 79–82, 2005.

[16] G. Vallancien, X. Cathelineau, H. Baumert, J. D. Doublet, and B. Guillonneau, "Complications of transperitoneal laparoscopic surgery in urology: review of 1,311 procedures at a single center," *Journal of Urology*, vol. 168, no. 1, pp. 23–26, 2002.

[17] E. S. Starling, L. O. Reis, R. Vaz Juliano et al., "Extraperitoneal endoscopic radical prostatectomy: how steep is the learning curve? Overheads on the personal evolution technique in 5-years experience," *Actas Urologicas Espanolas*, vol. 34, no. 7, pp. 598–602, 2010.

[18] J. U. Stolzenburg, R. Rabenalt, M. Do, F. Jiménez Cruz, and E. N. Liatsikos, "Laparoscopic extraperitoneal radical prostatectomy: evolution in time and updated results," *Actas Urologicas Espanolas*, vol. 30, no. 6, pp. 556–566, 2006.

[19] R. Katz, L. Salomon, A. Hoznek, A. D. La Taille, P. Antiphon, and C. Claude Abbou, "Positive surgical margins in laparoscopic radical prostatectomy: the impact of apical dissection, bladder neck remodeling and nerve preservation," *Journal of Urology*, vol. 169, no. 6, pp. 2049–2052, 2003.

[20] R. Bollens, M. Vanden Bossche, T. Roumeguere et al., "Extraperitoneal laparoscopic radical prostatectomy: results after 50 cases," *European Urology*, vol. 40, no. 1, pp. 65–69, 2001.

[21] J. Rassweiler, L. Sentker, O. Seemann, M. Hatzinger, and H. J. Rumpelt, "Laparoscopic radical prostatectomy with the Heilbronn technique: an analysis of the first 180 cases," *Journal of Urology*, vol. 166, no. 6, pp. 2101–2108, 2001.

[22] B. Guillonneau and G. Vallancien, "Laparoscopic radical prostatectomy: the Montsouris experience," *Journal of Urology*, vol. 163, no. 2, pp. 418–422, 2000.

[23] A. J. Vickers, C. J. Savage, M. Hruza et al., "The surgical learning curve for laparoscopic radical prostatectomy: a retrospective cohort study," *The Lancet Oncology*, vol. 10, no. 5, pp. 475–480, 2009.

[24] M. Ramirez Backhaus, J. Uwe Stolzenburg, M. Do, A. Dietel, J. L. Ruiz-Cerdá, and J. F. Jimenez Cruz, "Learning laparoscopic radical prostatectomy with the Leipzig program. Analysis of the training module program," *Actas Urologicas Espanolas*, vol. 33, no. 3, pp. 290–295, 2009.

[25] A. Raboy, G. Ferzli, and P. Albert, "Initial experience with extraperitoneal endoscopic radical retropubic prostatectomy," *Urology*, vol. 50, no. 6, pp. 849–853, 1997.

[26] M. Tobias-Machado, P. Forseto Jr., J. A. Medina, M. Watanabe, R. V. Juliano, and E. R. Wroclawski, "Laparoscopic radical prostatectomy by extraperitoneal access with duplication of the open technique," *International Brazilian Journal of Urology*, vol. 30, no. 3, pp. 221–226, 2004.

[27] S. M. Prasad, H. S. Maniar, N. J. Soper, R. J. Damiano, and M. E. Klingensmith, "The effect of robotic assistance on learning curves for basic laparoscopic skills," *American Journal of Surgery*, vol. 183, no. 6, pp. 702–707, 2002.

[28] J. P. Caballero Romeu, J. Palacios Ramos, J. G. Pereira Arias, M. Gamarra Quintanilla, A. Astobieta Odriozola, and G. Ibarluzea González, "Radical prostatectomy: evaluation of learning curve outcomes laparoscopic and robotic-assisted laparoscopic techniques with radical retropubic prostatectomy," *Actas Urologicas Espanolas*, vol. 32, no. 10, pp. 968–975, 2008.

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*Research Article*

# Inhibition of Androgen Receptor Expression with Small Interfering RNA Enhances Cancer Cell Apoptosis by Suppressing Survival Factors in Androgen Insensitive, Late Stage LNCaP Cells

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*Introduction*.The aim was to evaluate the changes of androgen receptor (AR) expression quantitatively and to identify influence of AR on cancer related survival markers in LNCap cell line. *Materials and Methods*. We compared expressions of AR, heat shock protein 27 (HSP27), clusterin (CLU), glucose-related protein 78 (GRP78), and cellular FLICE-like inhibitory protein (c-FLIP) and their genes between es-LNCaP (less than 33 times subcultured, L-33), ls-LNCaP (over 81 times subcultured, H-81), and si-LNCaP (AR siRNA transfected ls-LNCaP) by Western blotting and RT-PCR. *Results*. The expressions of AR, HSP27, CLU, GRP78, and c-FLIP were increased in ls-LNCaP compared with es-LNCaP (AR, 157%; HSP27, 132%; CLU, 146%; GRP78, 138%; c-FLIP, 152%). However, in si-LNCaP cell line, protein expressions were reversed to the level of es-LNCaP cell lines (25, 102, 109, 98, and 101%), and gene expressions on real-time PCR were also reversed to the expression level of es-LNCaP (ls-LNCaP: 179, 156, 133, 123, and 167%; si-LNCaP: 22, 93, 103, 112, and 107%). *Conclusions*. This finding suggests that androgen receptor can be related to the increased expression of cancer related survival markers such as HSP27, GRP78, CLU, and c-FLIP in late stage prostate cancer, and also inhibition of AR gene can be a therapeutic target in this stage of cancer.

## 1. Introduction

Nearly 29% of patients with newly diagnosed cancer in United States were diagnosed with prostate cancer which is the second most common cause of cancer death (11%, 22,720 patients) [1, 2].

At early stage, the prostate cancer is influenced markedly by androgen acting through the androgen receptor (AR) and, clinically, could be treated with surgical castration, radiation, or antiandrogen therapy. However, after initial response to these treatments, most androgendependent prostate cancer cells commonly progress to a highly aggressive, metastatic, androgenindependent state.

Androgen receptor (AR) is a 110 kDa phosphoprotein and one of the nuclear receptor superfamily of ligand activated transcription factors which elicits the biological response of androgens [3–5]. The AR is expressed in nearly all prostate cancer cells [6–8]. Growth and development of aggressive prostate cancer depend on androgen induced AR function [9–11].

Androgen independent prostate cancer development can be explained by the following five theories. (1) the AR hypersensitivity: under chemical castrated state induced by androgen ablation treatment, more AR is produced or the AR has enhanced sensitivity to androgen. (2) The promiscuous AR hypothesis: factors other than testosterone (i.e., estrogens, progestins, and antiandrogens) acts as a mutated AR agonists due to broaden specificity of AR. (3) The outlaw AR hypothesis: hormone independent prostate cancer growth or progress of AR independently through PTEN mutation and

activation of AR independent pathways such as PI3K and MAPK. (4) The bypass AR hypothesis: in androgen deprivation state, other antiapoptotic signal pathways through Bcl-2 overexpression and oncogene activation induce progression to HRPC. (5) The lurker cell hypothesis: androgen independent prostate cancer cells basically exist among epithelial stem cells even at androgen dependent state. After androgen deprivation, androgen independent malignant stem cells are selected to be activated [12].

LNCaP cell line is androgen sensitive human prostate cancer cells derived from the lymph node metastasis [13, 14]. Igawa et al. (2002) suggested the hormone sensitive LNCaP models changed to hormone independent cancer cells through long-term subcultures [15].

We focused on the factors related to the development of androgen independent prostate cancer. In previous studies using proteomic analysis, we confirmed that high passage subcultured LNCaP cells that acquired androgen independent property and the silencing of AR with small interfering RNA (siRNA) transfection resulted in the reversion of proteomic profile to level of es-LNCap cell line [16]. The aim of the present study was to evaluate changes of androgen receptor (AR) expression quantitatively and to identify influences of AR on cancer related proteins in LNCap cell line by comparing es-LNCaP and ls-LNCaP.

## 2. Materials and Methods

*2.1. Cell Culture and Experimental Groups.* LNCaP cells obtained from American Type Culture Collection (Bethesda, MD) were maintained in RPMI 1640 medium and made two LNCaP clones described in previous study [16]. All clones of LNCaP human prostate cancer cells were originated from the same source cell. The es-LNCaP cell was derived from low (less than 33) passage subculture and the ls-LNCaP, androgenindependent LNCaP, derived from high (more than 81) passage subculture. The si-ls-LNCaP subline was established by stably transfecting the ls-LNCaP cells with siRNA sequence. As control to silencing with siRNA, the scrambled siRNA, scr-ls-LNCaP was used.

*2.2. Doxazosin and siRNA Treatment.* Doxazosin (Sigma Aldrich Korea, Seoul, Korea) was prepared as described in previous study [17]. Cells were refed with fresh media at 80% confluence and treated with doxazosin or serum-free media containing 0.25% DMSO as control. The mRNA target sequences to AR (GeneBank Accession Number: NM000044) were designed using a siRNA template design tool (Ambion, Austin, TX), and siRNA was prepared with a Silencer siRNA construction kit (Ambion). Three oligonucleotides AR-1 (5′-GAC CUA CCG AGG AGC UUU CdTT-3′), AR-2 (5′-UCG AGG CCC UGU AAC UUG-3′), and AR-3 (5′-CAG UAG UUC GGA CAA ACG AAG A-3′) were designed based on the publicly released AR DNA sequence. The siRNAs were transfected into LNCaP cells with Lipofectamine 2000 (Invitrogen) employing 50 nM in 250 $\mu$L OptiMEM medium/60 mm culture dish. The transfected cells were allowed to grow for 24, 48, and 72 h at 37°C in a 5% $CO_2$ incubator and harvested for RT-PCR and immunoblot analysis. We performed immunohistochemical staining to confirm the expressions of AR in various LNCaP cells.

*2.3. Total RNA Extraction, Conventional RT-PCR, and Real-Time RT-PCR.* Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA). Cells ($5.0 \times 10^5$) were mixed in a test tube with 1 mL TRIzol solution. Prepared RNA was denaturated at 65°C for 15 min in a volume of 30 $\mu$L and cooled on ice for at least 1 min. 2.0 $\mu$g of denatured RNA were then annealed by addition of reaction mixture to a total volume of 20 $\mu$L (4.0 $\mu$L of 5 × RT buffer, 10 pmol of primers, 2.0 $\mu$L of 25 mM $MgCl_2$, 2.0 $\mu$L of 10 mM dNTPs, and 0.2 $\mu$L of 1 M DTT in nuclease-free water) and incubated at 42°C for 70 min. The reaction was terminated at 95°C for 5 min, chilled on ice for 5 min, and collected by brief centrifugation. To remove RNA, 1 $\mu$L of RNase H were added to each tube followed by incubation at 37°C for 20 min. 1 $\mu$L of cDNA were used for each PCR reaction.

Amplifications of cDNAs by PCR using specific primer pairs for AR were performed in 20 $\mu$L reaction volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 $\mu$M each dNTP, 0.2 $\mu$M of each primer, 1 unit of Taq DNA polymerase (Invitrogen, CA), and 1.0 $\mu$L cDNA as template.

Real-time PCR was performed with an SLAN real-time PCR detection system (LG Life science, Korea) and SYBR Green reagents (Invitrogen, Carlsbad, CA). Specific primers for human GAPDH, AR, HSP27, CLU, GRP78, and c-FLIP were designed to work in the same cycling conditions (50°C for 2 min to permit uracil N-glycosylase cleavage, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min). We used 1.0 $\mu$L of the reverse transcriptase product for PCR in a final volume of 25 $\mu$L.

*2.4. Western Blot.* Preparation of total cell lysate and the procedures for Western blot analyses were performed essentially as described previously [16]. The antibodies against GRP78, c-FLIP, and AR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for HSP27 purchased from Millipore (Millipore, MA). The quantity of the applied protein was normalized with anti-actin polyclonal antibody (Sigma Aldrich Korea, Seoul, Korea).

Samples with equal amounts of protein (20 $\mu$g) from lysates of cultured LNCaP cells were subjected to SDS-PAGE and then transferred to a PVDF filter. The filters were blocked in TBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with a diluted each primary antibodies (Actin: 1 : 10,000; AR, HSP27, CLU, GRP78 : 1 : 1,000; c-FLIP: 1 : 2,000; Santa Cruz, CA).

*2.5. Immunocytochemical Analysis and TUNEL Staining.* Cells on coverslips were rinsed 1 × phosphate-buffered saline (PBS) and then fixed with ice-cold methanol for 15 min. Samples were further permeabilized with PBS containing 0.025% Triton-X detergent (1 × PBS-TX) for 10 min and blocked with 3% BSA in 1×PBS for 30 min. Cells were reacted with primary antibodies (AR, HSP27, CLU, GRP78 : 1 : 100; c-FLIP: 1 : 50; Santa Cruz, CA) for 1 hour at room temperature. Cells were washed 3 times for 5 min with 1×PBS-TX and then incubated

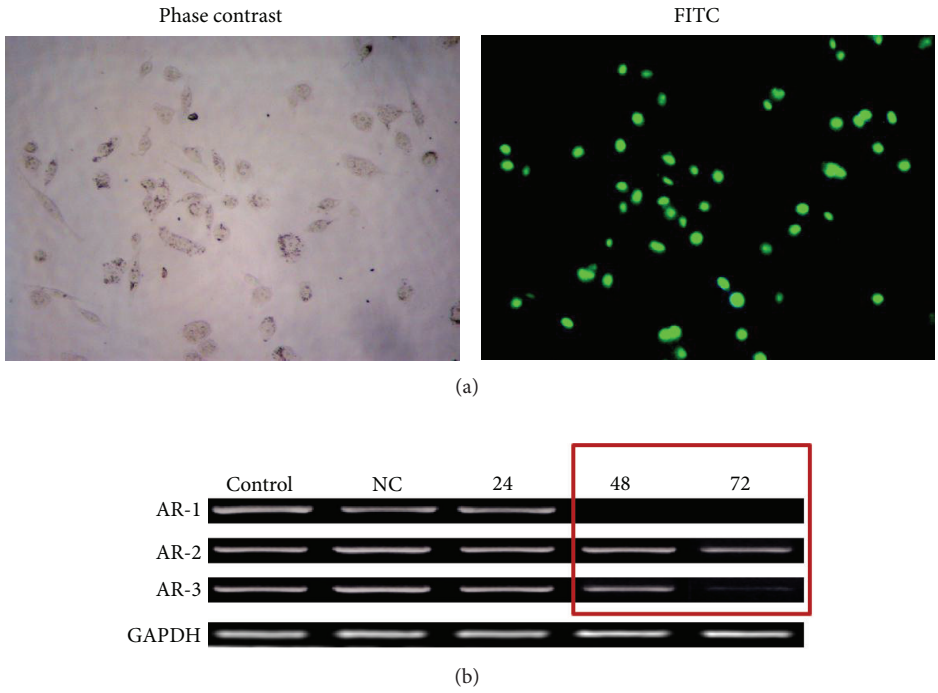


Figure 1: Effective silencing of the androgen receptor (AR) gene expression in ls-LNCap cells after small interfereing RNA (siRNA) treatment. (a) Transfection efficiency shown by fluorescence microscopy; (b) RT-PCR band of AR from si-ls-LNCap cells after siRNA. CNTL, untreated siRNA; NC, treated scrambled siRNA.

with horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, Santa Cruz. CA). Diaminobenzidine (DAB) was used as the chromogen and counterstaining was done with Mayers hematoxylin. Following three 5 min washes, cellscoverslips were mounted on slides with coverslips.

For TUNEL assays, fixed cells were incubated with an equilibrium buffer for 5 min using the in situ apoptosis detection kit, Fluorescein (Apoptag; Roche, BMS), and then treated in reaction buffer with 10 units of terminal deoxynucleotidyl transferase and 1 unit of deoxyuridine triphosphate digoxigenin at 37°C for 1 hour. The reaction was terminated by adding stop/wash buffer and then washed twice with Tris buffer. Anti-digoxigenin-FITC was added and reacted at 37°C for 30 min. After washing with distilled water, nuclei were counterstained with Hoechst 33258 (Sigma Chemical, St Louis, MO), and apoptosis in the cells was observed under a fluorescent microscope. Cells with green fluorescent (FITC) colored nuclei were considered apoptotic. For quantifying apoptotic cells, apoptotic and total cells were counted in 5 random fields scoring between 300 and 500 cells, and the numbers of apoptotic cells were expressed as percentages of the total cell population. Immunocytochemical staining slides and TUNEL staining slides were observed with microscope (TE-300, Nikon, Japan).

*2.6. Statistics Analysis.* Results were analyzed using a two-tailed Student's $t$-test to assess statistical significance. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

*3.1. Androgen Receptor siRNA Transfection Efficiency.* To explore the feasibility of siRNA transfection efficiency in knocking down AR expression in prostate cancer cells that harbor the AR gene, fluorescent oligo staining with BLOCK-iT was used and the green staining of more than 95% of cells was confirmed in siRNA transfected cells under fluorescence microscopy (Figure 1(a)). We designed and synthesized three siRNAs, AR-1, AR-2, and AR-3, against human AR gene. After 48 hours transfection with three sequence-specific siRNAs, one relatively potent siRNA, AR-1, was identified in knocking down AR expression compared with others by checking the mRNA with RT-PCR. This knocking down effect was sequence-specific event because a negative control siRNA with a scrambled sequence had no effect on AR expression level (Figure 1(b)).

*3.2. Immunocytochemistry of Markers.* Among the four experimental cell lines (es-LNCaP, ls-LNCaP, scr-ls-LNCaP, and si-ls-LNCaP), we confirmed the expression level of five prostate cancer related proteins (AR, HSP27, CLU, GRP78, and c-FLIP) with immunocytochemical staining (Figure 2). Positive staining of AR protein was more intensive in ls-LNCaP than es-LNCaP, but the expression level of AR protein in si-ls-LNCaP was almost disappeared (Figure 2). These results showed AR gene expression was almost inhibited by siRNA transfection. Moreover, the knocking down effect on the other cancer related proteins was also verified similarly.

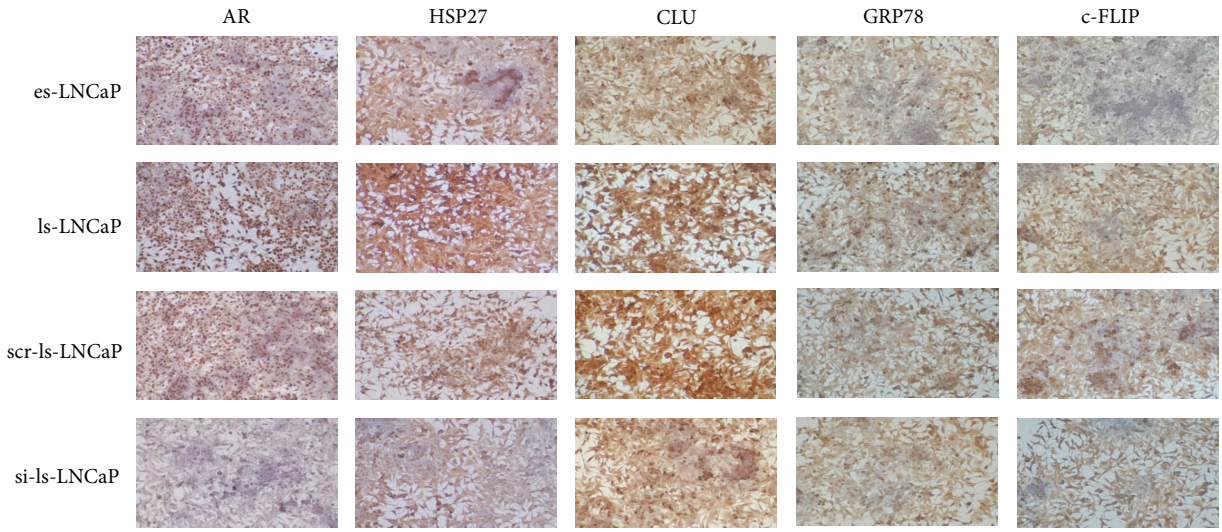


Figure 2: Immunocytochemical analysis of HSP27, clusterin, GRP78, and c-FLIP at the es-LNCap, ls-LNCap, scr-ls-LNCap, and si-ls-LNCaP cells.

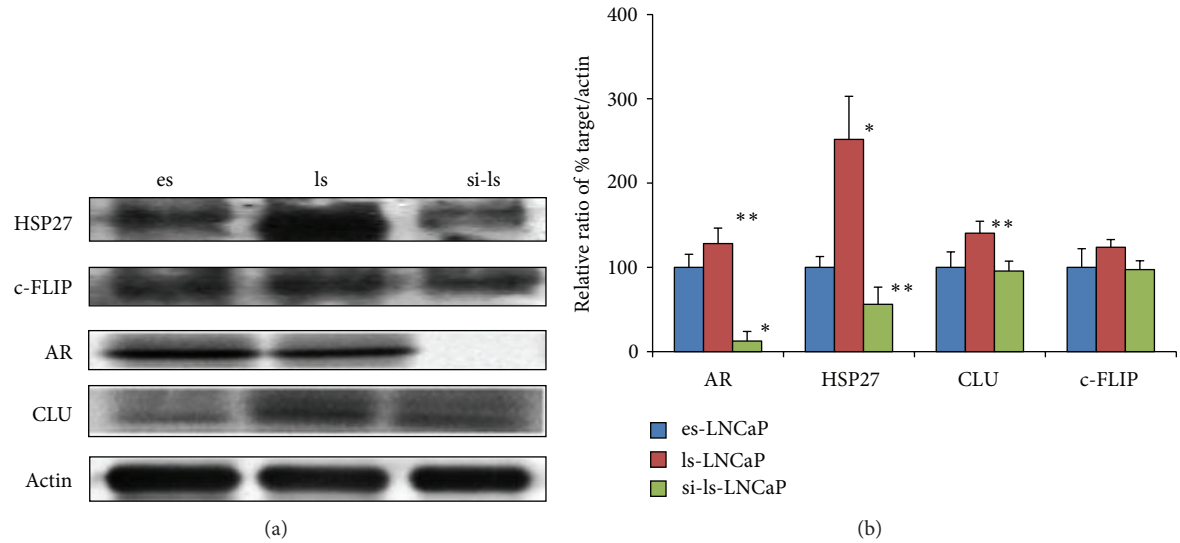


Figure 3: Electrophoretogram of immunoblot for androgen receptor, HSP27, c-FLIP, and clusterin expression at the es-, ls-, and the si-ls-LNCap cells.

The positive staining to HSP27, CLU, GRP78, and c-FLIP proteins in ls-LNCaP cells was more intensive than in es-LNCaP cells and was dramatically decreased in si-ls-LNCaP cells.

*3.3. Gene and Protein Expressions of Markers.* The expression of five prostate cancer related proteins (AR, HSP27, CLU, GRP78, and c-FLIP) increased in ls-LNCaP compared with es-LNCaP (AR, 157%; HSP27, 132%; CLU, 146%; GRP78, 138; and c-FLIP, 152%; Figure 3). But in si-ls-LNCaP cell line, protein expressions were decreased to level of es-LNCaP cell lines (25, 102, 109, 98, and 101%; Figure 3), and gene expressions on real-time PCR were decreased similarly (ls-LNCaP: 179, 156, 133, 123, and 167%; si-ls-LNCaP: 22, 93, 103, 112, and 107%; Figure 4).

*3.4. TUNEL Assay.* TUNEL assay was performed to see how doxazosin induced apoptosis was affected by the inhibition of AR gene (Figure 5). The number of TUNEL positive cells appeared less in ls-LNCaP cells compared to es-LNCaP counterpart. But, after AR was silenced, the number of TUNEL positive cells increased significantly.

## 4. Discussion

Prostate cancer cells are basically androgen dependent and androgen deprivation therapy (ADT) consistently causes prostate apoptosis and involution in first diagnosed prostate cancer. But, when prostate cancer advance further, it progresses into a more aggressive form of castration resistant prostate cancer (CRPC), refractory to all kinds of ADT.

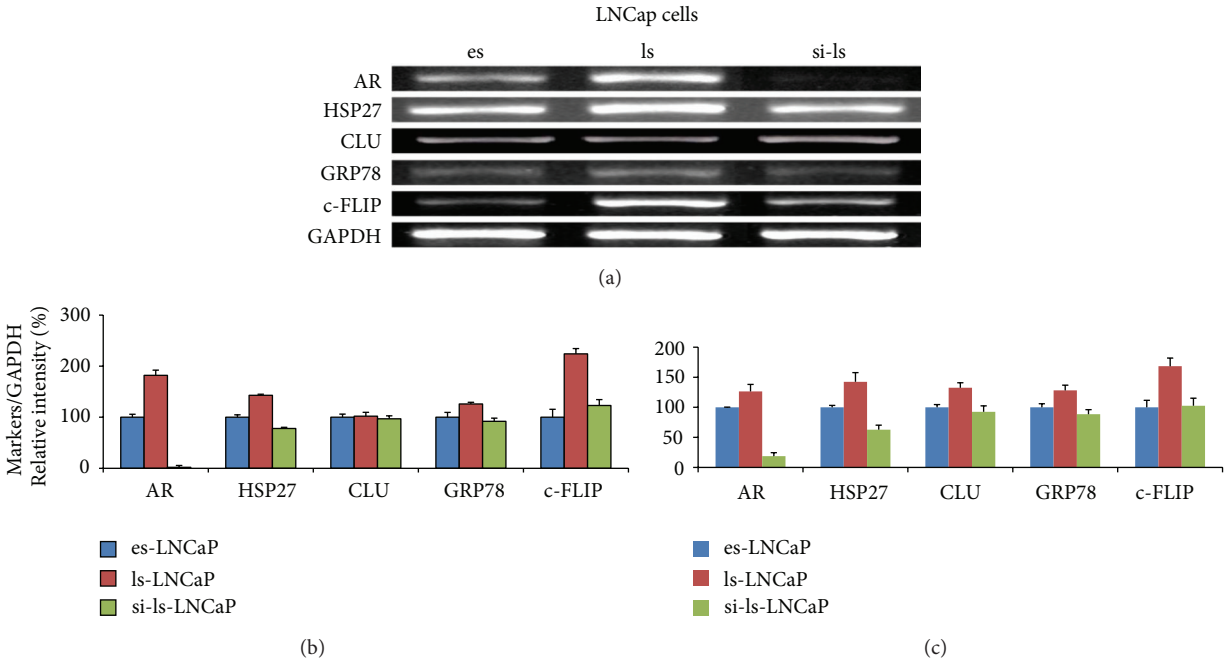


Figure 4: Electrophoretogram and its densitogram of conventional RT-PCR/real-time PCR product for androgen receptor, HSP27, CLU, GRP78, and c-FLIP expression at the es-, ls-, and the si-ls-LNCaP cells.

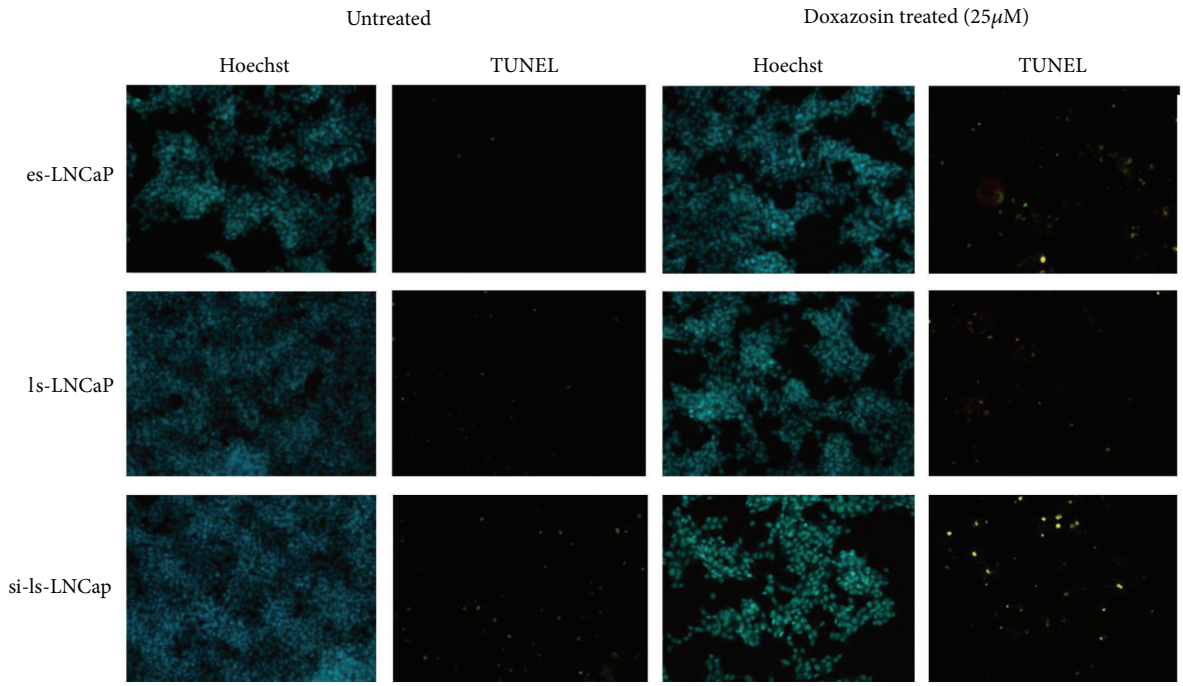


Figure 5: In situ detection of apoptotic cells in AR siRNA transfected cells at 48 hours after doxazosin treatment (25 $\mu$M). In situ detection of apoptotic cells in LNCaP cells was performed by $3'$-end labeling with digoxigenin-dUTP using terminal transferase.

Treatment of CRPC is very difficult and not that satisfactory so far. Docetaxel based chemotherapy is one of the most effective ways of treatments [18–20], but the overall survival benefit is only 2-3 months compared to conventional methods [21].

Diverse pathways have been discussed regarding progression to CRPC from androgen dependent counterpart. Among them, AR is considered having one of the most important roles with the possible mechanisms of hypersensitive AR or mutation of AR gene [12]. LNCaP cell lines are well known as

their androgen sensitive characteristics, but in our previous study, we showed the changes of cellular characteristics of early stage LNCaP cells (L-33) into androgen independent manner after long-term subculture (H-81) [16]. And we also found that there were significant differences in the expression of important survival antiapoptotic factors such as Tim, GRP78, and HSP27 using proteomics technique. We think that early and late stage LNCaP cell line model can help us to understand the mechanism of progression into the form of CRPC to some extent. In the present study, we showed higher level of AR mRNA and protein expression in H-81 compared to L-33, and this implicates that AR is closely related to the progression into H-81 characteristics via direct or indirect ways.

Among the possible mechanisms of chemotherapy failure of CRPC, several antiapoptotic factors such as c-FLIP [22], HSP27 [23], clusterin [17], and GRP78 [24] have been discussed. These factors have protective roles against apoptosis inducing stimuli. They are upregulated in various types of cancer cells and the degree of upregulation is proportional to the cancer aggressiveness. It also has been speculated that these survival factors help cancer cells to resist against various forms of anticancer treatment such as radiotherapy and cytotoxic chemotherapy.

Hsp27 suppresses apoptosis and probably has a critical role in progression to CRPC [25–29]. It has been reported that androgen insensitive LNCaP cells showed upregulation of HSP27 against androgen withdrawal and antiacancer drugs, such as paclitaxel [30].

GRP78 is a key member of the molecular chaperone heat shock protein (HSP) 70 family [31–33]. GRP78 expression is increased when AR expression is upregulated in LNCaP cells treated with DHT [34]. This is consistent with our findings in this study, showing further upregulation of GRP78 expression in H-81 cells.

Clusterin acts as an antiapoptotic factor and plays an important role in resistance to chemotherapeutic drugs [35]. When clusterin is overexpressed using vector transfection in rat prostate cell lines, transfected cells survived with blocking TNF-$\alpha$ induced apoptosis.

c-FLIP is also involved in apoptosis pathway regarding Fas signal transduction [22]. It is generally considered to have antiapoptotic roles in the prostate cancer [36]. c-FLIP expression is highly upregulated in the prostate cancer tissue when compared to normal tissue. It seems that maintaining high level of c-FLIP is essential and important in overcoming TNF related apoptosis in the prostate cancer [37]. It has also been known that transcription of c-FLIP is affected by AR [38].

Our study showed increased expression of clusterin, HSP27, GRP78, and c-FLIP in H-81 compared to L-33. mRNAs and proteins of these factors are downregulated below the levels of L-33 after AR knock-out using siRNA technique. Furthermore, the same concentration of doxazosin could induce more significant apoptosis after AR silencing. These observations suggest that Clusterin, HSP27, GRP78, and c-FLIP take part in the progression into H-81 in LNCaP model and AR is closely related to the upregulated expression and suppression of these survival factors. Our study also helps us to speculate that while prostate cancer cells become more aggressive with change of AR and overproduction of survival factors under the extremely stressful circumstances like androgen deprivation, these cells can be reverted into treatment sensitive traits when successful AR blocking causes suppression of various survival factors. It also supports the idea that therapeutic approach targeting AR can enhance the efficacy of anticancer treatment in the patients with metastatic CRPC, resisting against all forms of treatment.

Suppression of gene expression using siRNA is a simplified experimental technique which can regulate functions of specific factors at the gene level. AR silencing in the gene level is essential in the study of AR block, because AR is a transcription factor related to synthesis, regulation, and secretion of various kinds of proteins. In our present study, AR silencing successfully showed over 80% efficiency. We also showed higher level of discrimination using real time RT-PCR which enables us to find more significant differences between cell groups.

It can be summarized that in this LNCaP model, we observed that c-FLIP, HSP27, clusterin, and GRP78 take part in the progression into androgen insensitive status and the existence and overexpression of AR are closely related in this process. We think that these findings can be applied in the understanding of CRPC progression and treatment resistance of metastatic CRPC. New therapeutic approaches targeting AR regulation could be an effective solution against metastatic CRPC, currently incurable, and therefore various scientific efforts should be focused.

## Conflict of Interests

None of the contributing authors have any conflict of interests, including specific financial interests and relationships and affiliations relevant to the subject matter, materials, or methods discussed in the paper.

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## References

[1] R. Siegel, E. Ward, O. Brawley, and A. Jemal, "Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths," *CA Cancer Journal for Clinicians*, vol. 61, no. 4, pp. 212–236, 2011.

[2] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[3] D. J. Mangelsdorf and R. M. Evans, "The RXR heterodimers and orphan receptors," *Cell*, vol. 83, no. 6, pp. 841–850, 1995.

[4] D. J. Lamb, N. L. Weigel, and M. Marcell, "Androgen receptors and their biology," *Vitamins and Hormones*, vol. 62, pp. 199–230, 2001.

[5] S. M. Dehm and D. J. Tindall, "Molecular regulation of androgen action in prostate cancer," *Journal of Cellular Biochemistry*, vol. 99, no. 2, pp. 333–344, 2006.

[6] J. A. R. De Winter, P. J. A. Janssen, H. M. E. B. Sleddens et al., "Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer," *American Journal of Pathology*, vol. 144, no. 4, pp. 735–746, 1994.

[7] G. W. Chodak, D. M. Kranc, L. A. Puy, H. Takeda, K. Johnson, and C. Chang, "Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate," *Journal of Urology*, vol. 147, no. 3, pp. 798–803, 1992.

[8] M. V. Sadi, P. C. Walsh, and E. R. Barrack, "Immunohistochemical study of androgen receptors in metastatic prostate cancer: comparison of receptor content and response to hormonal therapy," *Cancer*, vol. 67, no. 12, pp. 3057–3064, 1991.

[9] C. W. Gregory, B. He, R. T. Johnson et al., "A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy," *Cancer Research*, vol. 61, no. 11, pp. 4315–4319, 2001.

[10] H. I. Scher and C. L. Sawyers, "Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis," *Journal of Clinical Oncology*, vol. 23, no. 32, pp. 8253–8261, 2005.

[11] M. E. Taplin and S. P. Balk, "Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence," *Journal of Cellular Biochemistry*, vol. 91, no. 3, pp. 483–490, 2004.

[12] B. J. Feldman and D. Feldman, "The development of androgen-independent prostate cancer," *Nature Reviews Cancer*, vol. 1, no. 1, pp. 34–45, 2001.

[13] T. Otsuka, K. Iguchi, K. Fukami et al., "Androgen receptor W741C and T877A mutations in AIDL cells, an androgen-independent subline of prostate cancer LNCaP cells," *Tumour Biology*, vol. 32, no. 6, pp. 1097–1102, 2011.

[14] F. Thomas, S. Patel, J. M. P. Holly, R. Persad, A. Bahl, and C. M. Perks, "Dihydrotestosterone SEnsitises LNCaP cells to death induced by epigallocatechin-3-Gallate (EGCG) or an IGF-I receptor inhibitor," *Prostate*, vol. 69, no. 2, pp. 219–224, 2009.

[15] T. Igawa, F. F. Lin, M. S. Lee, D. Karan, S. K. Batra, and M. F. Lin, "Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model," *Prostate*, vol. 50, no. 4, pp. 222–235, 2002.

[16] H. Y. Yun, S. Kim, Y. B. Young, and K. Y. Tag, "Proteomic analysis of androgen-independent growth in low and high passage human LNCaP prostatic adenocarcinoma cells," *Journal of Biochemistry and Molecular Biology*, vol. 41, no. 10, pp. 722–727, 2008.

[17] Y. H. Youm, H. Yang, Y. D. Yoon, D. Y. Kim, C. Lee, and T. K. Yoo, "Doxazosin-induced clusterin expression and apoptosis in prostate cancer cells," *Urologic Oncology*, vol. 25, no. 6, pp. 483–488, 2007.

[18] I. Marech, A. Vacca, G. Ranieri, A. Gnoni, and F. Dammacco, "Novel strategies in the treatment of castration-resistant prostate cancer (Review)," *International Journal of Oncology*, vol. 40, no. 5, pp. 1313–1320, 2012.

[19] C. J. Logothetis, E. Basch, A. Molina et al., "Effect of abiraterone acetate and prednisone compared with placebo and prednisone on pain control and skeletal-related events in patients with metastatic castration-resistant prostate cancer: exploratory analysis of data from the COU-AA-301 randomised trial," *The Lancet Oncology*, vol. 13, no. 12, pp. 1210–1217, 2012.

[20] D. Keizman, N. Maimon, and M. Gottfried, "Metastatic hormone refractory prostate cancer: recent advances in standard treatment paradigm, and future directions," *American Journal of Clinical Oncology*, 2012.

[21] J. Ansari, S. A. Hussain, A. Zarkar, J. S. Tanguay, J. Bliss, and J. Glaholm, "Docetaxel chemotherapy for metastatic hormone refractory prostate cancer as first-line palliative chemotherapy and subsequent re-treatment: birmingham experience," *Oncology Reports*, vol. 20, no. 4, pp. 891–896, 2008.

[22] H. Ye, Y. Li, J. Melamed et al., "Stromal anti-apoptotic androgen receptor target gene c-FLIP in prostate cancer," *Journal of Urology*, vol. 181, no. 2, pp. 872–877, 2009.

[23] C. Garrido, M. Brunet, C. Didelot, Y. Zermati, E. Schmitt, and G. Kroemer, "Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties," *Cell Cycle*, vol. 5, no. 22, pp. 2592–2601, 2006.

[24] H. Miyake, I. Hara, S. Arakawa, and S. Kamidono, "Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells," *Journal of Cellular Biochemistry*, vol. 77, no. 3, pp. 396–408, 2000.

[25] S. W. Lee, E. K. Kim, S. S. Kim, H. S. Uh, K. S. Cha, and T. K. Yoo, "Expression of heat shock protein 27 according to Gleason score and pathologic stage of prostate cancer," *Korean Journal of Urology*, vol. 50, no. 6, pp. 547–552, 2009.

[26] P. Rocchi, A. So, S. Kojima et al., "Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer," *Cancer Research*, vol. 64, no. 18, pp. 6595–6602, 2004.

[27] S. A. Thomas, "Detection and distribution of heat shock proteins 27 and 90 in human benign and malignant prostatic tissue," *British Journal of Urology*, vol. 77, no. 3, pp. 367–372, 1996.

[28] D. G. Bostwick, L. Klotz, and M. Garnick, "Immunohistochemical changes in prostate cancer after androgen deprivation therapy," *Molecular Urology*, vol. 4, no. 3, pp. 101–107, 2000.

[29] A. Valeri, R. Azzouzi, E. Drelon et al., "Heat-shock proteins inhibit induction of prostate cancer cell apoptosis," *Prostate*, vol. 45, no. 1, pp. 58–65, 2000.

[30] C. Andrieu, D. Taieb, V. Baylot et al., "Heat shock protein 27 confers resistance to androgen ablation and chemotherapy in prostate cancer cells through eIF4E," *Oncogene*, vol. 29, no. 13, pp. 1883–1896, 2010.

[31] J. Zhang, Y. Jiang, Z. Jia et al., "Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer," *Clinical and Experimental Metastasis*, vol. 23, no. 7-8, pp. 401–410, 2006.

[32] M. Shuda, N. Kondoh, N. Imazeki et al., "Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis," *Journal of Hepatology*, vol. 38, no. 5, pp. 605–614, 2003.

[33] G. Gazit, J. Lu, and A. S. Lee, "De-regulation of GRP stress protein expression in human breast cancer cell lines," *Breast Cancer Research and Treatment*, vol. 54, no. 2, pp. 135–146, 1999.

[34] S. S. Tan, I. Ahmad, H. L. Bennett et al., "GRP78 up-regulation is associated with androgen receptor status, Hsp70-Hsp90 client proteins and castrate-resistant prostate cancer," *Journal of Pathology*, vol. 223, no. 1, pp. 81–87, 2011.

[35] H. Miyake, C. Nelson, P. S. Rennie, and M. E. Gleave, "Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene Testosterone-repressed prostate message-2 in prostate cancer xenograft models," *Cancer Research*, vol. 60, no. 9, pp. 2547–2554, 2000.

[36] S. Gao, P. Lee, H. Wang et al., "The androgen receptor directly targets the cellular Fas/FasL-associated death domain protein-like inhibitory protein gene to promote the androgen-independent growth of prostate cancer cells," *Molecular Endocrinology*, vol. 19, no. 7, pp. 1792–1802, 2005.

[37] J. Zhang, N. Gao, S. Kasper, K. Reid, C. Nelson, and R. J. Matusik, "An androgen-dependent upstream enhancer is essential for high levels of probasin gene expression," *Endocrinology*, vol. 145, no. 1, pp. 134–148, 2004.

[38] D. B. Longley, T. R. Wilson, M. McEwan et al., "c-FLIP inhibits chemotherapy-induced colorectal cancer cell death," *Oncogene*, vol. 25, no. 6, pp. 838–848, 2006.

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*Research Article*

# Promising Noninvasive Cellular Phenotype in Prostate Cancer Cells Knockdown of Matrix Metalloproteinase 9

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Cell surface interaction of CD44 and MMP9 increases migration and invasion of PC3 cells. We show here that stable knockdown of MMP9 in PC3 cells switches CD44 isoform expression from CD44s to CD44v6 which is more glycosylated. These cells showed highly adhesive morphology with extensive cell spreading which is due to the formation of focal adhesions and well organized actin-stress fibers. MMP9 knockdown blocks invadopodia formation and matrix degradation activity as well. However, CD44 knockdown PC3 cells failed to develop focal adhesions and stress fibers; hence these cells make unstable adhesions. A part of the reason for these changes could be caused by silencing of CD44v6 as well. Immunostaining of prostate tissue microarray sections illustrated significantly lower levels of CD44v6 in adenocarcinoma than normal tissue. Our results suggest that interaction between CD44 and MMP9 is a potential mechanism of invadopodia formation. CD44v6 expression may be essential for the protection of non-invasive cellular phenotype. CD44v6 decrease may be a potential marker for prognosis and therapeutics.

## 1. Introduction

Prostate cancer is the third most common cause of death from cancer in men. Prostate cancer is a disease of extensive metastases with secondary lesions in lymph nodes, brain, bones, and sometimes in visceral organs such as the liver and lungs. Prostate cancer patients initially respond to androgen ablation therapy. However, prolonged androgen ablation therapy results in relapse and androgen independent prostate cancer progression with bone metastasis. Bone metastasis occurs in 90% of patients with advanced stage prostate cancer. The advanced stage of prostatic carcinoma eventually metastasizes to the bones in 85–100% of cases.

Adhesion of breast and prostate cancer cells to the bone marrow endothelial cell line is directly related to the surface expression of the hyaluronic acid (HA) receptor CD44 which is a transmembrane glycoprotein [1, 2]. CD44 binds with HA through its amino-terminal conserved region [3]. CD44 functions as a protein responsible for cellular attachment to the extracellular matrix (ECM), migration, invasion, and apoptosis [1, 4–7]. The molecular mass of conserved CD44 termed CD44-standard (CD44s) is about 85–90 kDa. This is the product of transcription of exons 1–5 and 16–20. Exons 6–15 encode for separate CD44 variant isoforms from CD44v1 (not expressed in human cells) to CD44v10 [8]. The amino terminal region also contains several sites for O-linked glycosylation and attachment to chondroitin sulphate [3]. Posttranslational glycosylation of different CD44 variants produce proteins with molecular mass ranging from 80 to 200 kDa [4].

The biological role of the CD44 molecules is not the same in all tumors. Along with CD44s, one or multiple splice variants may be expressed in cancer cells displaying an increased tendency for expressing larger isoforms; for example, expression of CD44v8-10 in pancreatic carcinomas [9] and CD44v6 in colorectal cancer [10] and prostate cancer [11, 12]. CD44 has been suggested to play a role in the metastatic spread of prostate cancer cells [13, 14]. However, reduced and heterogeneous expression of CD44v6 was shown in six cases of primary prostate cancer by immunohistochemistry

analysis [11]. The decreased expression of CD44s has also been shown to be involved in the progression of prostate cancer to a metastatic state [15]. The role of CD44 in prostate cancer development and progression remains obscure and needs further elucidation.

CD44s surface expression and CD44s/matrix metalloproteinase 9 (MMP9) interaction on the cell surface are associated with secretion of active MMP9 and migration/invasion of PC3 cells [1]. Disruption of CD44/MMP9 interaction on the cell surface reduces migration and invasion of PC3 cells. MMP9 knockdown of PC3 cells showed reduced CD44 at cellular and surface levels [12]. An increase in the formation of invadopodia and localization of MMP9 in invadopodia may possibly increase the invasive characteristic of PC3 cells [1, 6]. The addition of a neutralizing antibody to CD44s reduced active MMP9 at the cell surface and secreted levels. Surface expression of CD44 and activation of MMP9 on the cell surface are interdependent [1, 12]. The reciprocal activities of the two proteins on the cell surface reveal an interesting situation that poses the question, "What is the biological implication of their interaction?" We hypothesize that CD44/MMP9 proteins contribute to the high metastatic property through the formation of invadopodia.

To address this question, we generated stable PC3 cell lines deficient in MMP9 and CD44 by RNA interference knockdown method. Downregulation of MMP9 expression switches CD44 isoform expression from CD44s to CD44v6 which is more glycosylated. These cells attain the phenotype of noninvasive cells as a result of failure in the formation of invadopodia. Expression and glycosylation of CD44v6 is accompanied with extensive cell spreading and adhesion which is due to the formation of focal adhesions and stress fibers in these cells. Our data suggest that downregulation of MMP9 increases the adhesive and noninvasive phenotype in PC3 cell through the expression of CD44v6. CD44 knockdown reduces adhesive and survival properties of PC3 cells in a time-dependent manner.

## 2. Materials and Methods

*2.1. Materials.* Antibodies to GAPDH, actin, and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to CD44s and Streptavidin-HRP were purchased from Cell Signaling Technology (Danvers, MA). Antibodies specific to CD44v6 and CD44v10 were purchased from R&D Systems (Minneapolis, MN), EMD Biosciences (Gibbstown, NJ) and Bender Medsystems, Inc. (Burlingame, CA). Rhodamine phalloidin and all chemicals reagents were purchased from Sigma-Aldrich (St. Louis, MO). Matched normal tissue and tumor tissue lysates made from single person were purchased from Abcam (Cambridge, MA).

*2.2. Cell Lines Used for Studies and Culture Conditions.* We have used metastatic carcinoma-derived cell lines which include: (i) PC3, from skeletal metastases [16, 17]; (ii) LNCaP from lymph nodes [18]; and (iii) DU-145 from brain [16]. These cell lines were obtained from American Type Culture Collection (Manassas, VA). Normal prostate epithelial (HPR-1) [19, 20] and benign prostatic hyperplasic (BPH) cells [21–23] have been used as controls. We generated stable MMP9 and CD44 knockdown PC3 cells lines using respective SiRNA or ShRNA constructs as described previously [12, 24]. Stable PC3 cell lines expressing control scrambled RNAi were used as controls. MMP9 (PC3/Si) and CD44 (PC3/Si (CD44)) knockdown PC3 cells are denoted as indicated in parentheses.

Prostate cancer cell lines and benign prostatic hyperplasic control cells (BPH) were maintained in RPMI1640 (Gibco BRL, Life Technologies, Bethesda, MD) containing 5 or 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin as described previously [1]. Normal prostatic epithelial cells (HPR-1) were cultured in keratinocyte (serum-free) medium supplemented with EGF (2.5 mg/500 mL), bovine pituitary extracts (25 mg/500 mL; Gibco BRL, Life Technologies, Bethesda, MD), and 1% penicillin/streptomycin [19].

*2.3. Quantitative Real-Time RT-PCR Analysis.* Quantitative real-time RT-PCR (qPCR) was performed using an Applied Biosystems Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) as described previously [25]. Primer sequences and PCR product size (in parenthesis) are as follows: hCD44s (129 bp)—forward 5′ACCGACAGCACAGACAGAATC3′; reverse 5′GTTTGCTCCACCTTCTTGACTC3′; hCD44v6 (149 bp)—forward 5′GCAGCACTTCAGGAGGTTACAT3′, reverse 5′GGTAGCTGTTTCTTCCGTTGTA3′; hCD44v10 (149 bp)—forward 5′GCAGCACTTCAGGAGGTTACAT3′; reverse 5′ATGATTTGGGTCTCTTCTTCCA3′; GAPDH (132 bp)—forward 5′CTTTGGTATCGTGGAAGGACTC3′; reverse 5′GTAGAGGCAGGGATGATGTTCT3′. All reactions were prepared in triplicate and four independent sets of samples were used in each experiment.

*2.4. Analysis of Cell Surface Expression of CD44v6 by Biotinylation and Flow Cytometry.* Cells were washed with PBS and labeled with NHS-biotin according to the manufacturer's guidelines (Pierce, Rockford, IL). In conjunction with immunoprecipitation and immunoblotting analyses, the levels of surface labeled proteins were determined as described previously [1]. Flow cytometry analysis (FACs analysis) was performed essentially as described previously [26].

*2.5. Migration and Invasion Assays.* Wound healing and phagokinesis assays were done as described previously [26, 27]. For invasion assays, cross-linked fluorescein isothiocyanate (FITC)-conjugated gelatin matrix-coated cover slips were prepared as described [6]. To assess the formation of invadopodia and degradation of FITC-gelatin matrix, cells were cultured on FITC-gelatin-coated cover slips for 12–14 h as shown previously [6]. Cells were fixed and stained for actin with rhodamine phalloidin as described previously [27]. Gelatin matrix and actin-stained cells were viewed and photographed with a Bio-Rad confocal laser-scanning microscope. Images were stored in TIF format and processed by using Photoshop (Adobe Systems, Inc., Mountain View, CA).

*2.6. Deglycosylation of Proteins.* Deglycosylation of CD44v6 protein was done as previously described [28]. About 500 $\mu$g protein was vacuum dried and resuspended in 500 $\mu$L of trifluoromethanesulfonic acid (TFMSA; SIG-158534) and 1/10th to 1/2 volume of anisole (SIG-96109). The suspension was incubated for 2–6 h on ice and the reaction was stopped with cold N-ethylmorpholine (SIG-04499; 4 : 1 volume). TFMSA treatment was done in three tubes for 2, 4 and 6 h to determine the time dependent effect on complete deglycosylation. 5–10 volumes of acetone (Merck) was added to the tube and mixed well. The mix was incubated overnight at −20°C and centrifuged for 10 min at 10000 rpm to pellet protein. The pellet was dried and resuspended in SDS-containing sample buffer (100 $\mu$L) prior to SDS-PAGE and immunoblotting with an antibody to CD44v6. Immunoblotting was done as previously described [27].

*2.7. Immunostaining.* Surface localization of CD44 (CD44s, CD44v6 and CD4v10) and MMP9 was determined in cells that were not permeabilized with Triton X-100. Cells fixed for 5 min with paraformaldehyde (3.7%), washed twice with cold PBS, and blocked with blocking solution were used for immunostaining with antibodies of interest as described previously [27]. Immunostained cells were viewed and photographed with a Bio-Rad confocal laser-scanning microscope. Images were stored in TIF format and processed by using Photoshop (Adobe Systems, Inc., Mountain View, CA).

*2.8. Immunohistochemistry.* Prostatic cancer and normal tissue microarray (TMA) sections with stage and grade information were bought from US Biomax, Inc. (Rockville, MD). We have used the following tissue microarray sections containing different number of cases and cores: PR242 (12 cases with 24 cores); PR481 (24 cases with 48 cores) and PR956 with metastasis in bone and alimentary wall (40 cases with 95 cores). Sections were arranged in duplicate cores per case. TMA sections were processed, stained, and analyzed essentially as described previously [24, 29]. Images were taken with an Aperio scanscope CS system (Vista, CA). Relative distribution of interested proteins in immunostained TMA sections were semiquantitatively analyzed by two other investigators.

*2.9. Statistical Analysis.* All values presented as mean ± SEM. A value of $P < 0.05$ was considered significant. Statistical significance was determined by analysis of variance (ANOVA) with the Bonferonni corrections (Instat for IBM; Graph pad software).

## 3. Results

*3.1. MMP9 Knockdown Increases Expression of CD44v6.* PC3 cells express CD44 isoforms such as CD44s, v6, and v10. MMP9 knockdown in PC3 cells (PC3/Si) reduces the expression of CD44s [12]. Here, we evaluated the expression levels of CD44v6 and v10 at RNA and protein levels in PC3/Si cells. As shown previously, MMP9 knockdown reduced the expression of CD44s at mRNA (Figure 1(a)) and protein levels (data not shown). However, real time RT PCR and immunoblotting analyses (Figures 1(a)–1(d)) displayed a significant increase in the expression of CD44v6 and v10. The increase in mRNA was found to be >3–5 fold for CD44v6 and ~1–1.5 fold for CD44v10 in PC3/Si cells as compared with vector (PC3/V) and a scrambled SiRNA construct (PC3/Sc) transfected PC3 cells (Figure 1(a)).

Immunoblotting analysis showed equal levels of CD44v6 protein at molecular weight (MW) 80–85 kDa in all PC3 cells lines tested including PC3/Si (Figure 1(b)). In addition to this 80–85 kDa protein, several protein bands of CD44v6 with MW ranging between 80 and >150 kDa were observed in these cells lines. However, these bands were significantly increased in PC3/Si than PC3, PC3/V and PC3/Sc cells. An increase in the mRNA of CD44v10 (Figure 1(a)) does not appear to be translated into CD44v10 protein in PC3/Si cell line (Figure 1(c)). Equal levels of CD44v10 protein with a MW ~115 kDa was observed in all PC3 cell lines tested (Figure 1(c)). The correlation between increased mRNA and decreased protein levels of CD44v10 remains unclear. It is possible that CD44v10 may not have any functional significance in prostate cancer cells.

CD44v6 expression is essentially restricted to a subset of epithelia in nonmalignant tissues [30]. Therefore, we compared the expression levels of CD44v6 in normal prostatic epithelial (HPR1) and benign prostatic hyperplasic (BPH) cells with LNCaP, DU145 prostate cancer cells (Figure 1(e)). The amount of CD44v6 in BPH and HPR1 cells (Figure 1(e)) is as good as PC3/Si cells (Figure 1(b)). Expression of CD44v6 (~80 kDa) was observed in DU145 cells but at a significantly lower level (Figure 1(e), DU; indicated by an arrow). We failed to detect CD44v6 in LNCaP cells (LN). Taken together, our results demonstrate a switch in the CD44 isoform expression from CD44s to CD44v6 in PC3/Si cells. Expression of CD44v6 in PC3/Si cells as observed in HPR1 and BPH cells suggest that downregulation of MMP9 has the potential to reverse the malignant phenotype of PC3 cells.

*3.2. MMP9 Knockdown Increases Surface Expression and Glycosylation of CD44v6*

*3.2.1. Fluorescence Activating Cell Sorting (FACs) Analysis.* Next, we analyzed the surface expression levels of CD44v6 in PC3/Si and control cells using flow cytometry analysis and subsequently assessed again by biotinylation procedure. A representative histogram analysis for CD44v6 is shown in Figure 2(a). A shift in the fluorescence histogram indicates an increase in the surface levels of CD44v6 in PC3/Si cells (Figure 2(a), peak 1) as compared with control cells (PC3, PC3/V, and PC3/Sc cells). Figure 2(b) is a bar graph quantitation showing a ~50–60% increase in PC3/Si cells. We were able to corroborate this observation in the immunoblotting analysis with lysates made from indicated cells surface labeled with NHS-biotin (Figure 2(c)).

As shown in Figure 1, several protein bands of CD44v6 with MW ranging between 80 and >150 kDa were observed in PC3/Si cells. The level of these protein bands was significantly more in PC3/Si cells (Figure 2(c), lane 4) than control PC3

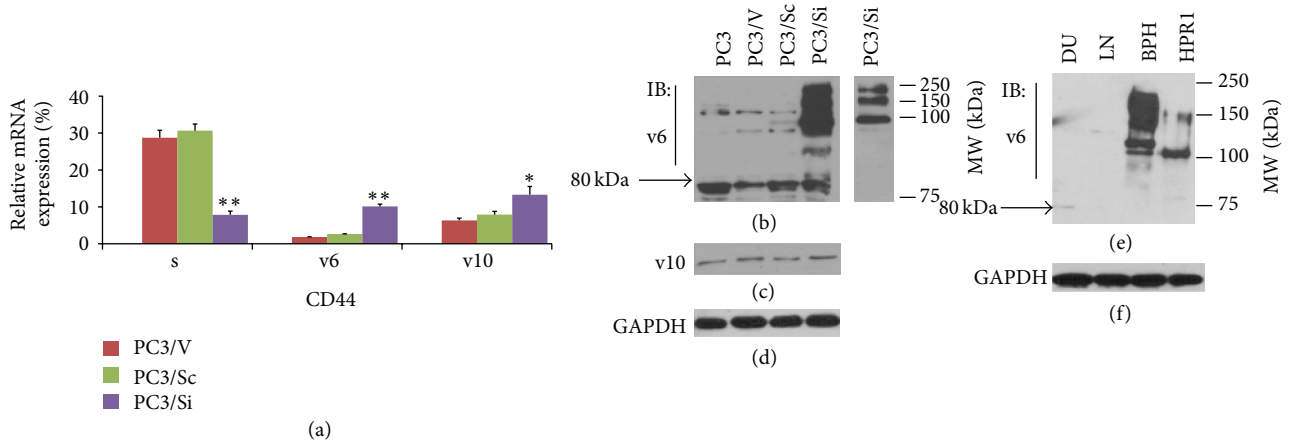


Figure 1: Determination of the expression of CD44s, v6, and v10 in PC3 cells knockdown of MMP9: PC3 cells transfected with vector DNA (PC3/V), scrambled nonsilencing SiRNA (PC3/Sc) and silencing SiRNA (PC3/Si) were used for real time PCR (a) and immunoblotting analyses (b–f). Untransfected PC3 cells were also used as controls. The expression levels of CD44s, CD44v6, and CD44v10 mRNA were determined by real time PCR analysis and normalized relative to GAPDH expression (a). $^{**}P < 0.001$; $^{*}P < 0.01$ versus indicated respective controls (PC3/V and PC3/Sc). Equal amount of protein was used for immunoblotting analysis with an antibody to CD44v6 (b and e) and CD44v10 (c). A shorter exposure blot for PC3/Si cells is shown in lane 5. The blot in (b) was stripped once and probed successively with an antibody to CD44v10 (c) and GAPDH (d). The blot in (e) was stripped and reprobed with an antibody to GAPDH. The levels of GAPDH represent the loading control for each experiment set. The results shown are representative of three or four experiments.

and PC3/Sc cells (lanes 2 and 3). A shorter exposure blot for PC3/Si is shown in lane 5. The blot was stripped and reprobed with an antibody to Zip1 (d). Zip1 was used as a loading control for surface proteins. It is a cell surface zinc transporter protein and was shown to express ubiquitously on the surface of PC3 cells [23]. We have previously demonstrated that osteoclasts derived from bone marrow cells express only CD44s [31]. To determine the specificity of CD44v6 antibody, we used CD44s (data not shown) and nonimmune IgG (Figure 2(c), lane 1) immunoprecipitates made from lysates of osteoclasts and PC3/Si cells, respectively. Immunoblotting analysis showed no detectable levels of CD44v6 due to nonspecific binding (Figure 2(c), lane 1) further validating the specificity of the CD44v6 antibody.

*3.2.2. Deglycosylation of by TFMSA.* CD44v6 contain a number of potential glycosylation sites which may explain its migration at a higher molecular weight. Trifluromethanesulphonic acid (TFMSA) was shown to deglycosylate proteins and produce predicted size peptides from cDNA [28]. Therefore, in order to determine the degree of glycosylation, total cellular lysate protein (~500 $\mu$g) was deglycosylated with (+) and without (−) TFMSA (Figure 2(e)). Immunoblotting analysis with an antibody to CD44v6 demonstrated that TFMSA reduces the protein size to ~80–85 kDa implying that Posttranslational modification (glycosylation) of CD44v6 is mediated by glycosyltransferases. The actual mechanisms of glycosylation in response to MMP9 knockdown and the glycosyltransferases which regulate glycosylation of CD44v6 have yet to be determined.

*3.2.3. Immunostaining and Confocal Microscopy Analysis.* To determine the surface distribution of indicated proteins, immunostaining was done in cells not permeablized with Triton X-100 (Figure 3). As shown previously [1], colocalization (yellow) of CD44s and MMP9 was observed on the cell surface of PC3/Sc cells (Figure 3(a"); Overlay). The expression of CD44s is reduced in PC3/Si cells (Figures 3(d') and 3(d")), [1]. Therefore these cells (d) exhibited reduced colocalization of CD44s and MMP9 on the cell surface (d"). Surface distribution of v6 (b' and e') and v10 (c') in PC3/Sc and PC3/Si cells corroborates immunoblotting and FACs analyses shown in Figure 2. Phase contrast microscopy analysis showed that PC3/Si cells undergo a dramatic morphological alteration in cell size and shape as compared with PC3/Sc cells. PC3/Si cells are larger in size [12]. Punctate and patchy distribution of CD44v6 on the cell surface indicates that this protein is abundantly expressed by PC3/Si cells (e' and e"). Interestingly, the size of the spots appears to be larger than that observed in the control group (b'). Punctate and patchy staining indicates micro clustering of CD44v6 on the cell surface and in the periphery of plasma membrane (indicated by arrows in e"). This suggests that CD44v6 may possibly have different cellular function other than cell invasion. It is possible that CD44v6 mediated signaling may increase the adhesive nature and provide a widespread morphology to PC3/Si cells as well. Taken together, for the first time, we show here that down-regulation of MMP9 is accompanied by the upregulation CD44v6 at transcriptional, translational (total and surface levels of protein) and Posttranslational (glycosylation) levels.

### 3.3. MMP9 Knockdown Reduces Migratory and Invasive Property

*3.3.1. Migration Assays.* Having established that MMP9 knockdown increases the expression of CD44v6 in PC3 cells,

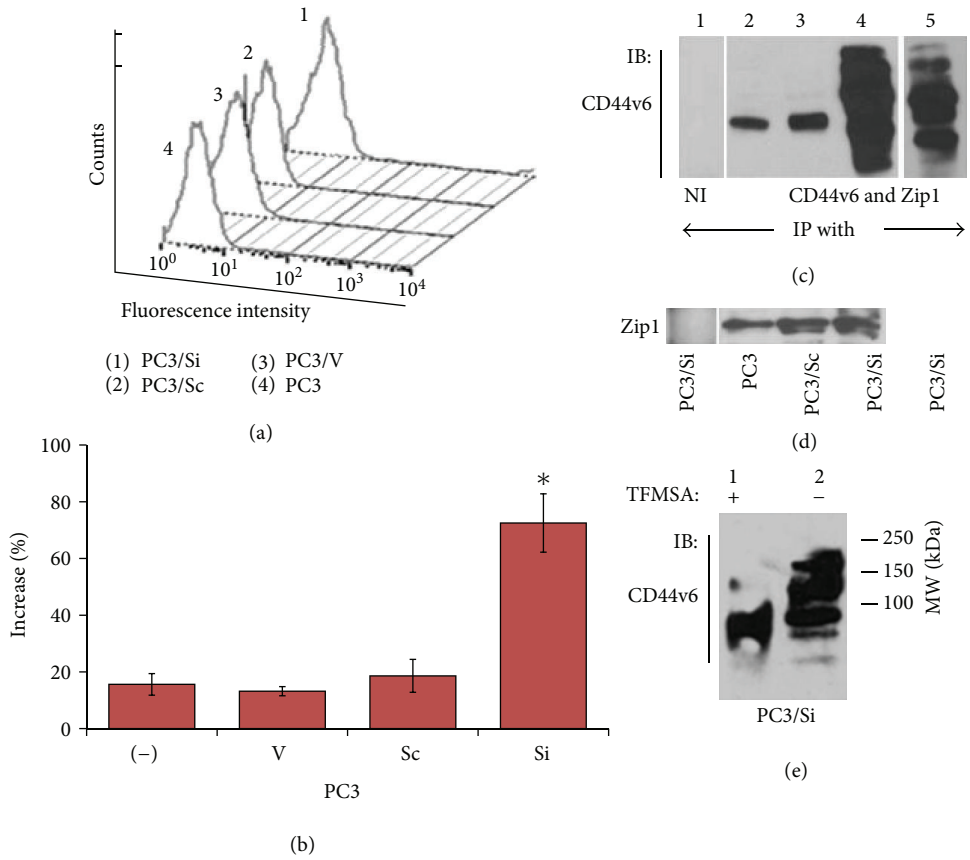


Figure 2: Analysis of surface expression of CD44v6 in indicated PC3 cell lines. (a) and (b), Surface expression of CD44v6 in PC3 cells knockdown of MMP9 (PC3/Si) is compared with control cell lines (PC3, PC3/V, and PC3/Sc) by FACs analysis (a) and (b). A representative histogram demonstrating the surface levels of CD44v6 in various PC3 cell lines is shown. In a typical experiments about 4–6 wells in 24 wells were used for each cell line. Statistical analysis of the mean fluorescence intensity (mean ± SEM; $n = 3$) is shown as a graph (b); $^{*}P < 0.0001$ versus control cell lines. (c) and (d) Immunoblotting analysis of surface expression of CD44v6 and Zip1 proteins in indicated PC3 cell lines. Equal amount of lysate proteins (100 $\mu$g) were immunoprecipitated with an antibody to CD44v6 (c) and Zip1 (d) and pulled down with streptavidin agarose. The blot was probed with a CD44v6 antibody followed by a Zip1 antibody after membrane stripping. A shorter exposure blot for PC3/Si is shown in lane 5. (e) Deglycosylation of CD44v6 with TFMSA. Total lysates (500 $\mu$g protein) made from PC3/Si cells were treated with (+) and without (–) TFMSA. Immunoblotting analysis demonstrates the deglycosylated (lane 1) and non-deglycosylated (lane 2) CD44v6 (e). The results shown are representative of three different experiments.

we next examined the functional consequence of this change in cell migration (Figure 4) and invasion (Figure 5) assays. Cell migration was assessed by phagokinesis (a and b) and wound healing (d–h) assays. In phagokinesis assay, PC3/Si cells displayed a significant decrease in migration (Figures 4(b) and 4(c)) as compared with PC3/Sc cells (a and c). Similar observations were made in the wound healing assay. A decrease in the wound size from 48.6 ± 8 $\mu$m at 0 h (Figure 4(d)) to 16.2 ± 3 $\mu$m at 24 h (Figure 4(f)) was observed in PC3/Sc cells. However, the wound size was 47.9±7 $\mu$m at 0 h (Figure 4(e)) and 43.3 ± 5 $\mu$m at 24 h (Figure 4(g)) in PC3/Si cells. Statistical analysis is provided as a graph at 0 h, 12 h, and 24 h (Figure 4(h)). Wound healing is comparable in PC3 and PC3/Sc cells. These cells move toward the wound and the wound area decreased over time. However, MMP9 knockdown reduces or delayed wound closure significantly. The defect in migration is reflected in the morphology and size of PC3/Si cells (Figure 4(g); [12]).

*3.3.2. Invasion Assays.* Next, we proceeded to check the invasive property of PC3/Si cells using gelatin degradation assay as shown previously [12]). Cells were stained with rhodamine phalloidin for actin (red) and analyzed by a confocal laser scanning microscopy (Figure 5). PC3/Sc cells displayed actin staining in several distinct invadopodia-like structures (indicated by arrow heads in Figure 5(a)). Degradation of FITC-conjugated gelatin matrix (green) was observed (Figure 5(b), indicated by green arrows) and PC3/Sc cells were found within the excavated matrix (Figure 5(a)). However, PC3/Si cells failed to demonstrate matrix degradation (c). A discrete reorganization of actin cytoskeleton with the formation stress fibers and focal adhesions was observed in PC3/Si cells (Figures 5(c) and 5(d); indicated by arrows). Consistent with our observations in gelatin degradation invasion assay, MMP9 knockdown significantly reduced 3D extracellular matrix degradation and 3D invasion in an OrisTM Cell Invasion & Detection Assay system containing 3D surface (see Figure S1

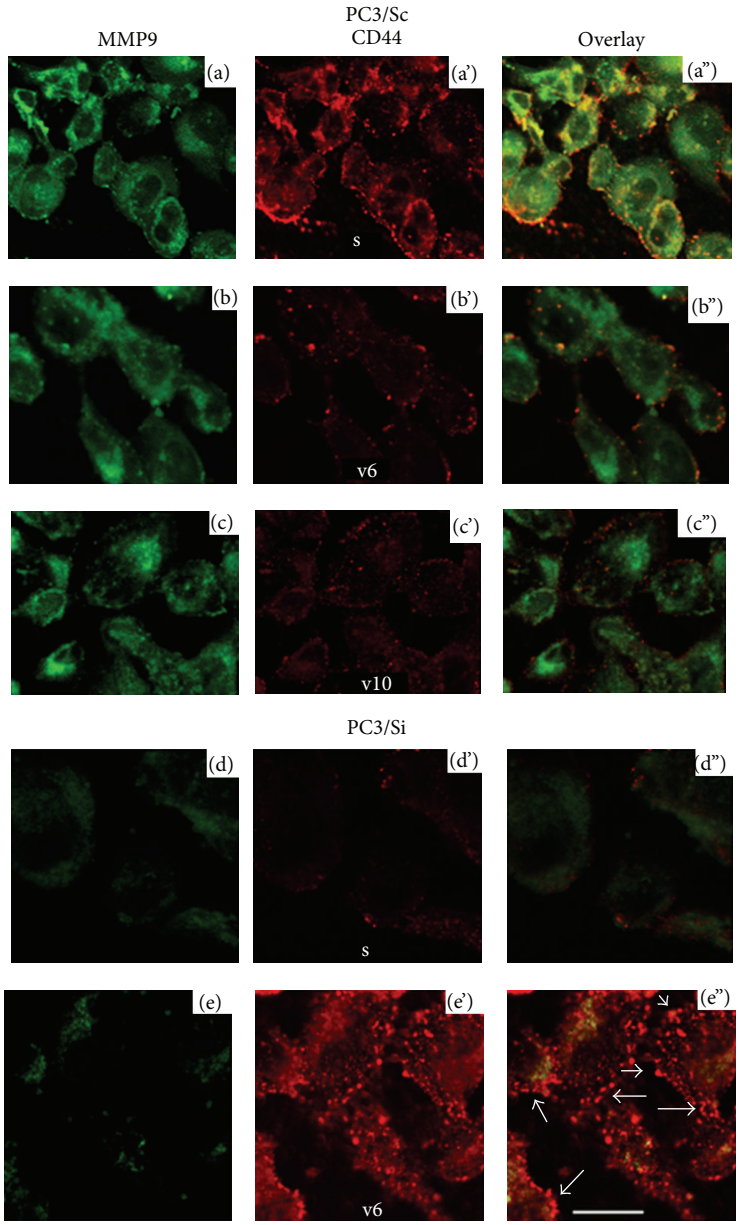


Figure 3: Immunostaining and confocal microscopy analysis of surface distribution of MMP9 and indicated CD44 isoforms in PC3/Sc and PC3/Si cell lines. Immunostaining was performed with antibodies to MMP9 (green) and isoforms of CD44 (s, v6, and v10; red). Distribution of both MMP9 and indicated CD44 isoforms is shown in overlay panels (a")–(e"). Yellow color indicates colocalization of CD44s and MMP9 on the cell surface (overlay panel a"). Scale bar-50 $\mu$m. The results represent one of three experiments performed.

avaliable online at http://dx.doi.org/10.1155/2013/493689). These results confirmed the dependence of prostate cancer cells migration and invasion on MMP9 activity and the formation of invadopodia.

*3.4. CD44 Knockdown Reduces Adhesive Property of PC3 Cells.* Since MMP9 and CD44s has interdependent role in the invasion of PC3 cells, the next logical step is to understand whether knockdown of CD44 in general will have any impact on the organization of invadopodia in PC3 cells. Four different silencing and one control scramble ShRNA constructs for the CD44s cDNA sequences (Genbank-NM_000610.3; encodes the longest isoform) were made to knockdown CD44 in PC3 cells as described previously [24]. PC3 cells stably transfected with vector DNA and a scrambled nonsilencing ShRNA construct were used as controls. Several individual clones (~15–20) were characterized for each construct and tested the expression levels of CD44s. A significant decrease in CD44s was observed in the clonal isolates of PC3 cells transfected with silencing ShRNA constructs corresponding to nucleotide sequences 492 and 801 bp [24]. Among the individual clones tested, we chose clones which demonstrated very minimal or no expression of CD44s for further studies

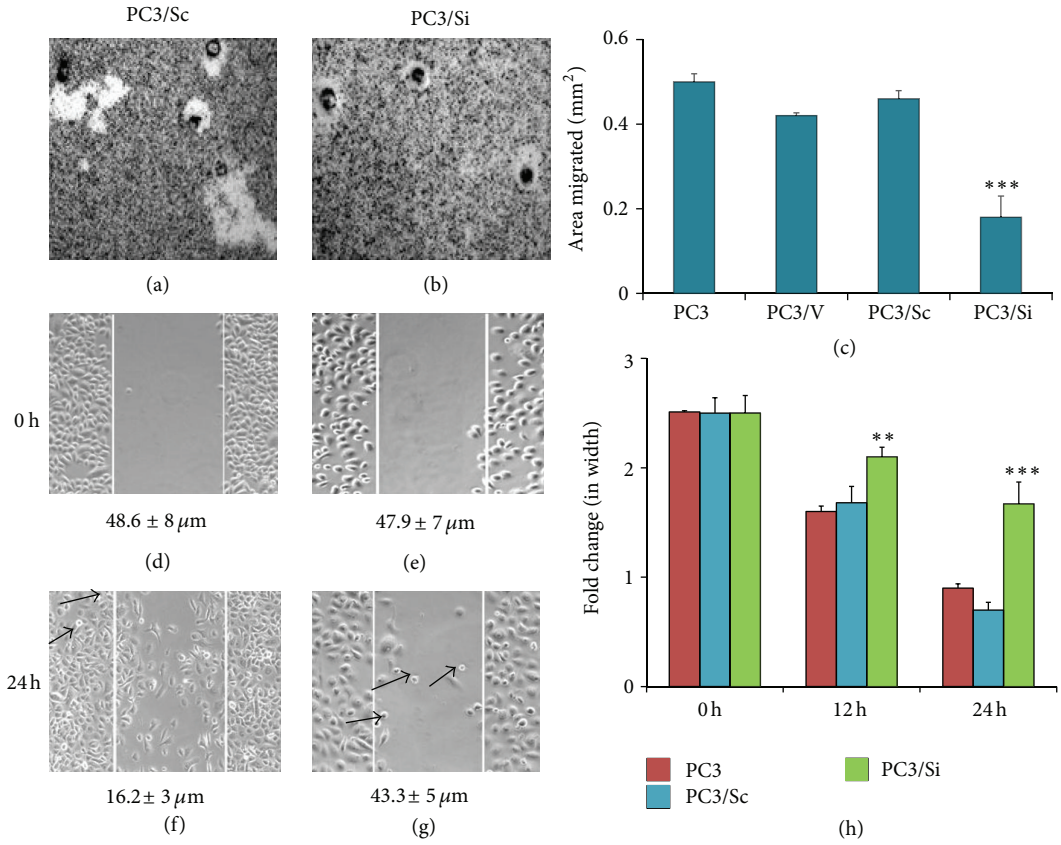


Figure 4: The effects of MMP9 knockdown on the migration of PC3 cells. Phase contrast micrographs of PC3/Sc and PC3/Si cells are shown (a) and (b); (d)–(g). (a)–(c) Phagokinesis assay. The area of the migratory track is seen free of the gold particles. PC3 cells were seen as black spots in the white tracks (a) and (b). Photographs were taken at the same magnification (100× in the lower-power view). The motility was evaluated by measuring areas free of gold particles and represented as $mm^2$ (c). Results shown are mean ± SEM of three independent experiments. About 20 to 25 cell tracks from each experiment were measured; $^{***}P < 0.001$ versus control cells (PC3, PC3/V, and PC3/Sc). (d)–(h) Wound closure assay. A representative wound closure assay before (d) and (e) and after (f) and (g) migration of PC3/Sc (d) and (f) and PC3/Si (e) and (g) is shown. Arrows in (f) and (g) point to dead floating cells. Data showed at the bottom of each panel (in $\mu$m) represent mean ± SEM of an experiment performed in triplicates at 24 h. Statistical analysis (mean ± SEM) of three experiments performed in triplicates at 12 and 24 h is provided as a graph (h). A significant decrease in the migration and wound closure was observed at 12 and 24 h in PC3 cells knockdown of MMP9 (PC3/Si) as compared with indicated control cells. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control cells (PC3/Sc and PC3/V).

(Figure 6(a), lanes 1 and 2). These cells failed to exhibit the basal level expression of CD44v6 as well (data not shown).

The morphological changes in PC3 cells by knockdown of CD44 (PC3/Si (CD44) were assessed by phase contrast microscopy. The morphology of PC3 cells is shown in Figure 6(c). PC3/Si (CD44) cells were smaller in size and membrane bleb-like projections were seen at the periphery after the cells were adhered to cell culture dishes for 24 h (Figure 6(e)). These changes in the morphology match up with the reorganization of actin filaments into retraction of cell protrusions with the formation of numerous cytoplasmic processes or microvilli-like structures at the periphery. Neither invadopodia nor stress fibers/focal adhesions were observed in these cells (Figure 6(f)). Retraction of cell protrusions and the formation of cytoplasmic processes could be a sign of rounding up of cells as a result of reduced CD44s signaling. This may be one of the characteristic features of cell detachment. Therefore, cells were cultured for 72 h and viewed under phase contrast microscope. Cells rounded up and detached at 48 h and 72 h in a time dependent manner. Only a few cells were attached to cell culture dishes at 72 h. Cell viability is reduced after 5 days in culture (data not shown). We present evidence that CD44 signaling can control invadopodia formation and cell adhesion in PC3 cells. Knockdown of CD44 in general has the potential to reduce the invasive and survival property of PC3 cells [32].

*3.5. Expression of CD44v6 Is Low in Prostatic Adenocarcinoma in Tissue Microarray Sections.* In order to confirm the expression levels of CD44v6 in multiple prostate cancer tissue samples from autopsy, we carried out immunohistochemistry analyses in prostate cancer tissue microarray (TMA) sections containing matched normal adjacent tissue (Figure 7). Number of cases and cores in the TMA are indicated in Materials and Methods. A representative tissue microarray panel containing sections of normal and tumor tissue (24 cores) and

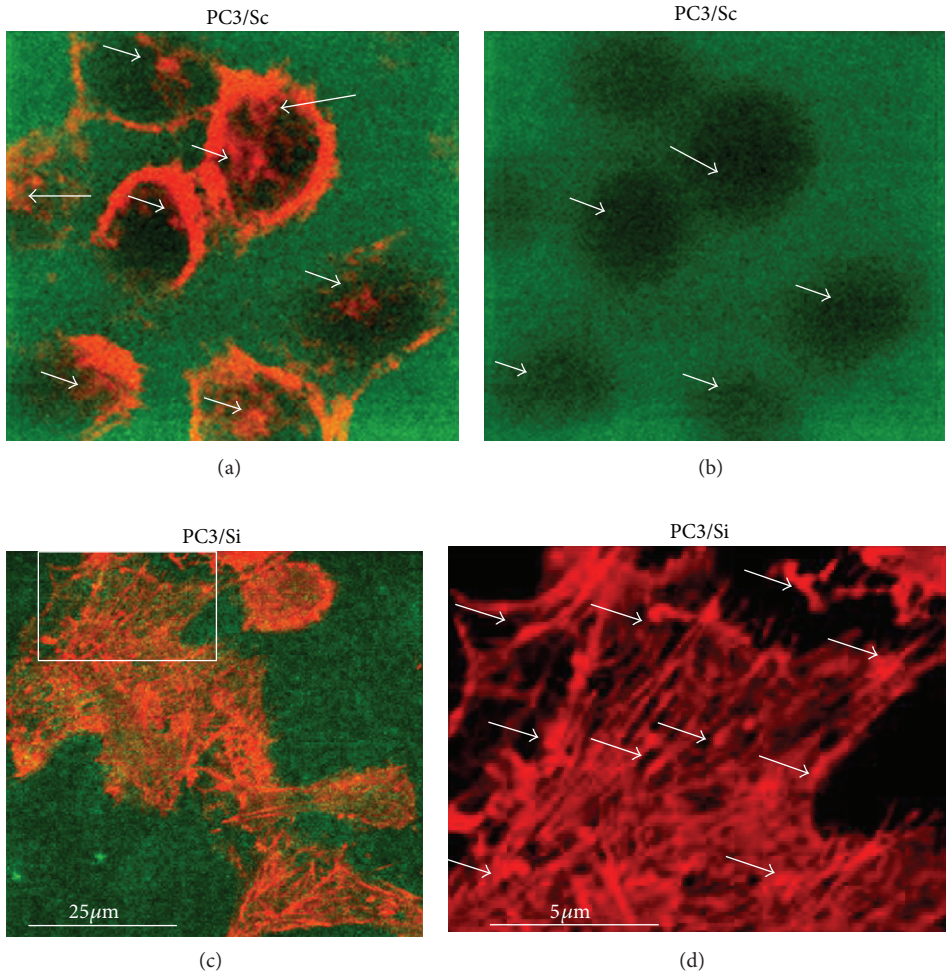


Figure 5: Determination of invasion property of PC3/Si and PC3/Sc cells using gelatin degradation assay. The ability of PC3 cells knockdown of MMP9 to degrade the gelatin matrix was determined by culturing PC3 cells (PC3/Sc and PC3/Si) on a FITC-conjugated gelatin matrix (green). Cells were found within the degraded matrix (a). Actin staining is shown in red (a), (c), and (d). Invadopodia are indicated by arrow heads (a). Areas of degradation of the matrix are indicated by green arrows (b). The rectangular field in (c) is enlarged in (d). Focal adhesions are indicated by arrows (d). These results represent one of several experiments performed.

the cores which are selected to show at higher magnification are indicated by a rectangular field in Figure S2 (additional file). Also, semiquantitative analysis distribution of CD44v6 in normal prostatic tissue, prostatic adenocarcinoma (stage 3 and 4), and metastatic adenocarcinoma in bone is shown in Figure S3 (additional file). Medium (a–f) and higher (a'–f') power view of indicated core in Figure S2 are shown for the specificity of staining in Figure 7.

Normal prostate tissue showed positive staining for CD44s and MMP9 in the cytoplasm and nuclei of luminal epithelial and basal cells (Figures 7(c) and 7(e)). Prostatic adenocarcinoma sections showed multiple small foci of tumor cells (d and f). One of the foci is showed in higher magnification (d' and f'). Overexpression of CD44s (d and d') and MMP9 (f and f') was clearly observed in tumor cells. CD44v6 expression is more in normal tissue (a) than in tumor tissue (b). High power view (a') demonstrated intense staining in the basal cells and basolateral plasma membrane (indicated by arrows in a') of luminal epithelial cells. Prostatic adenocarcinoma at stages 1–4 showed weak reactivity for CD44v6 (b and b'). The foci of tumor cells in the same core or tissue section which demonstrated intense staining for CD44s (d') and MMP9 (f') displayed minimal staining for CD44v6 at the periphery in the monolayer cells (b'). No staining was observed in the tumor cells found within the foci (b'). Similarly, prostatic adenocarcinoma at stages 1–4 as well as prostate cancer metastasis to bone and abdominal wall showed weak reactivity for CD44v6 (Figure S3 in additional file). Taken together, these observations imply that there is a switch in the expression of CD44v6 to CD44s occurs during metastatic process. We suggest here that downregulation of CD44v6 is related and perceived to be important in the prognosis and progression of prostate cancer. Expression of CD44s may serve as molecular marker for invasiveness.

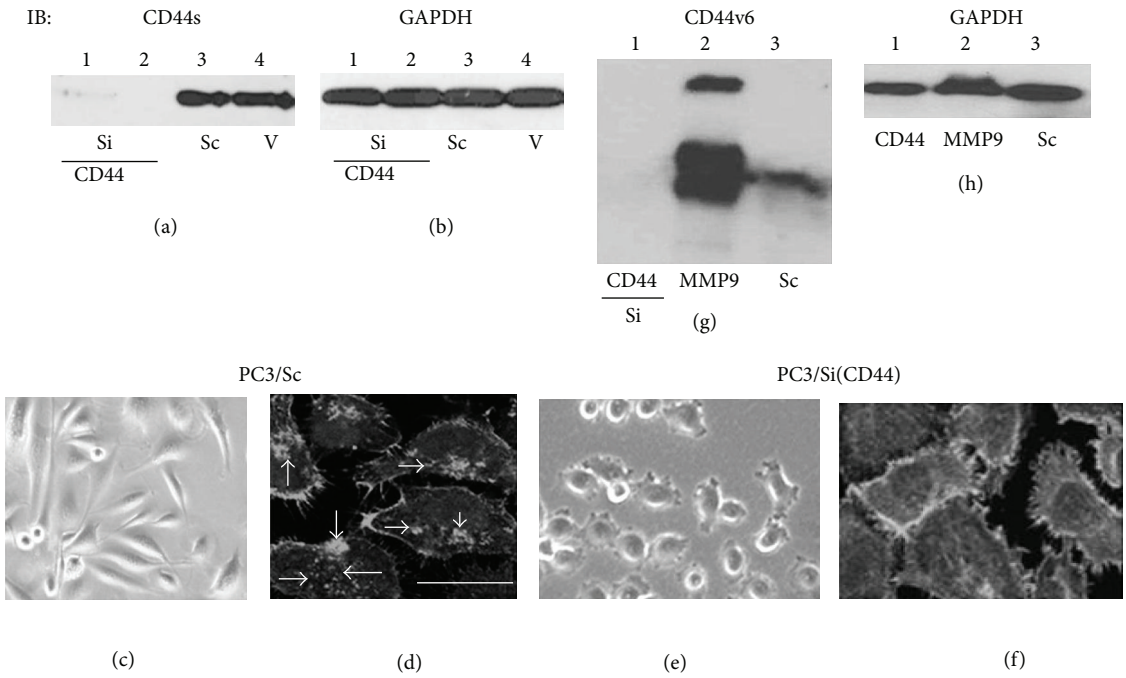


Figure 6: Analysis of the effects of CD44 knockdown on the expression of CD44v6, cell morphology, and actin distribution. (a), (b), (g), and (h) The effect of ShRNA to CD44 on the cellular levels of CD44s protein was determined by immunoblotting analysis. The expression levels of CD44s are shown in different PC3 cell lines by immunoblotting analysis (a). Individual clones were isolated from cells transfected with a construct corresponding to 492 bp ((a), lane 1) and 801 bp (lane 2). Cells transfected with a nonsilencing scrambled ShRNA construct (Sc; lane 3) and vector DNA (V; lane 4) were used as controls. PC3 cells knockdown of CD44s ((g), lane 1) and MMP9 (lane 2) as well as a control PC3/Sc cells (lane 3) were used for immunoblotting analysis with an antibody to CD44v6. Total cellular lysates (~50 $\mu$g) were used for immunoblotting analyses (a), (b), (g), and (h). Equal loading of protein was verified by immunoblotting with an antibody GAPDH (b) and (h). The experiment was repeated three times with similar results. (c)–(f) The morphology of PC3/Sc and PC3/Si (CD44) cells was determined by phase contrast microscopy (400x; (c) and (e)). Cells were stained with rhodamine phalloidin to examine the distribution of actin in PC3/Sc (d) and PC3/Si (CD44) (f) cells. Staining of actin is shown in gray scale (d) and (f). Arrows point to invadopodia in PC3/Sc cells (d). The results shown are representative of three independent experiments. Bar: 50 $\mu$m.

## 4. Discussion

The aim of this study is to understand the underlying molecular mechanisms regulating invasion in prostate cancer cells. Highly invasive cancer cells expressing invadopodia degrade and enter the matrix produced by cells like fibroblasts [33–35]. Normal cells neither form invadopodia nor degrade the ECM [36]. We have shown here and previously that localization of MMP9 in the invadopodia of PC3 cells regulates the invasion into the matrix [6]. To elucidate the possible roles of MMP9 in prostate cancer cell invasion/migration processes, we generated PC3 cells knockdown of MMP9 (PC3/Si). PC3/Si cells adhered and spread well in culture [12]. Invasion analyses revealed that PC3/Si cells are noninvasive in nature which is certainly due to failure in the formation of invadopodia.

CD44 expression has been associated with aggressive behavior of various tumor cells [13, 37, 38]. Src, Rho, cortactin, WASP, and Arp2/3 proteins are involved in the formation of invadopodia [6, 33, 39, 40]. Failure to form invadopodia in PC3 cells treated with a WASP peptide suggests that actin polymerization and formation of invadopodia involves the WASP-Arp2/3 complex [6]. Several of these important proteins which induce invadopodia formation (e.g., cortactin, Src, Rho, and WASP) were shown to interact with CD44 [41, 42]. PC3/Si cells are noninvasive in nature which is not only due to failure in the formation of invadopodia but also due to reduced expression of CD44s. However, downregulation of MMP9 expression in PC3 cells switches CD44 isoform expression from CD44s to CD44v6 which is more glycosylated. This is accompanied with the formation of focal adhesions and stress fibers in PC3/Si cells. Focal adhesions have been shown to have both adhesive and invasive properties in pancreatic cancer cells [43]. An increase in the expression and glycosylation of CD44v6 isoform in PC3/Si cells possibly suggest a role for CD44v6 signaling in the formation of focal adhesions and stress fibers. CD44 isoforms may have unique signaling properties. It seems that the biological role of CD44 molecules is not the same in all cell types or tumors.

The expression of different CD44 isoforms has been correlated with the human tumor progression (rev. in [44]). In addition to a strong expression of CD44s, variant isoforms of CD44 (CD44v) were observed predominantly in aggressive lymphoma. This has been associated with a shorter overall survival of patients [45]. In a rat metastasis model,

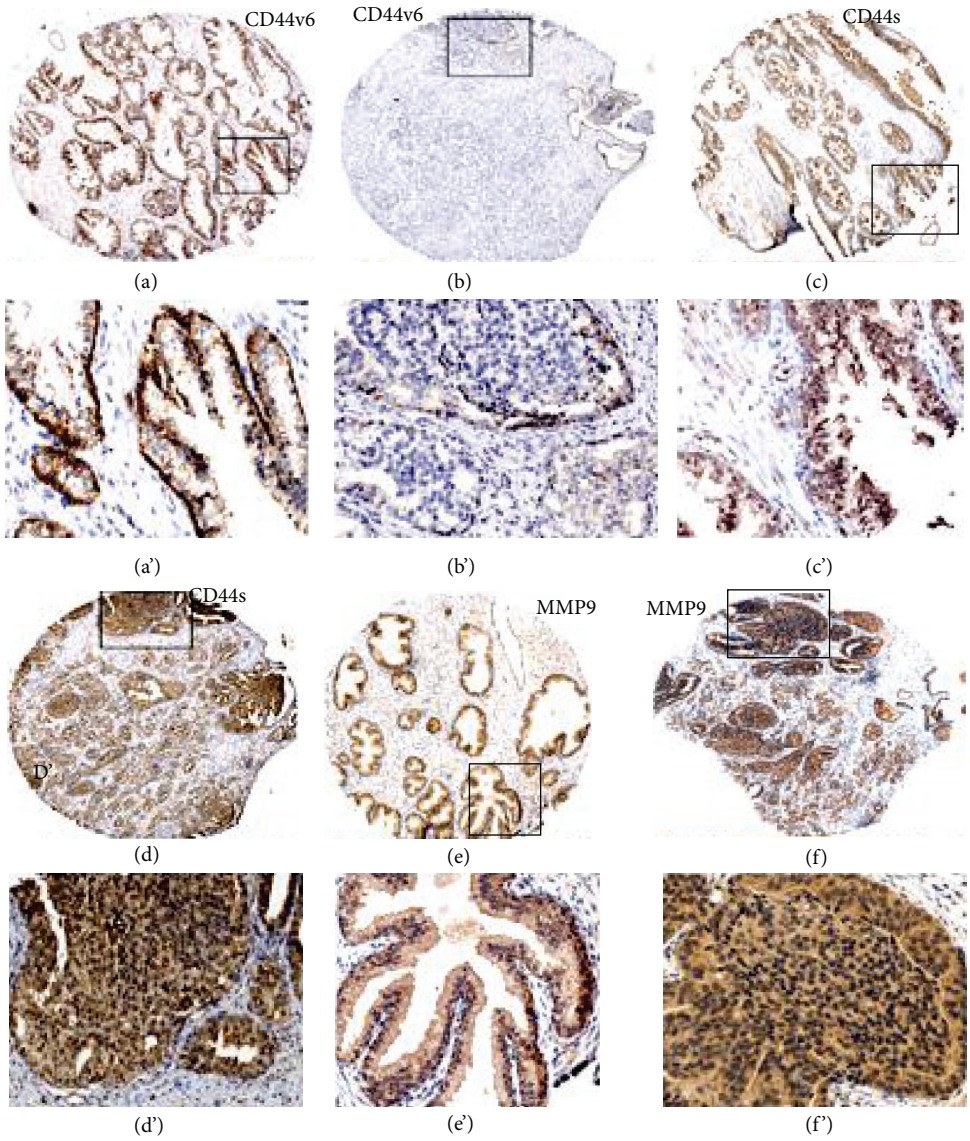


Figure 7: Immunohistochemical detection of CD44v6 (a) and (b), CD44s (c) and (d), and MMP9 (e) and (f) in normal (a), (c), and (e) and adenocarcinoma (grade 2-3; (b), (d), and (f)) sections of representative prostate tissues from a tissue microarray: immunostained sections (brown) with indicated antibody above were counterstained with hematoxylin stain (blue). Note the intense immunopositivity of basal epithelial membrane of epithelial cells in normal gland (indicated by arrows in (a')). Magnification is 50× in (a)–(f). Location of the high magnification (200×) regions shown in (a')–(f') is indicated by a rectangle field in (a)–(f). The staining was repeated thrice with similar results.

v6 isoform is causally involved in lung metastasis formation [44]. CD44v6 isoform appears to be the major importance for tumor dissemination in human non-Hodgkin's lymphoma, colon carcinoma and mammary carcinoma [46–49]. Down-regulation of MMP9 has an impact on the expression and surface levels of CD44s. However, the intriguing observation is the expression and glycosylation of CD44v6 in PC3/Si cells corresponds with the benign (BPH) and normal prostatic (HPR1) epithelial cells. As shown by others in cases of carcinoma [50], we have shown here that the expression of CD44v6 is down regulated in prostate cancer tissue. Expression of CD44 variant isoforms containing sequences are tightly regulated and restricted to epithelial cells [45, 51, 52]. Expression of CD44v6 in HPR1 and BPH cells corroborate this observation. It is possible that PC3/Si cells may assume a noninvasive epithelial-like phenotype which may trigger the expression of CD44v6. It suggests that CD44 isoforms can exert different molecular mechanisms and functions depend on the situation. Thus, unlike what is observed in other tumors, CD44v6 appears to be inversely positive with tumor progression in prostate cancer.

The role of CD44 isoforms in prostate cancer development and progression needs further elucidation. Evidence of CD44 involved in prostate cancer is conflicting as some have shown increased expression of CD44s and CD44 variants to be associated with poor prognosis. However, others found

a reduction of CD44 variants in tumors of patients with advanced carcinoma. With the use of PC3/Si and PC3/CD44s cell lines, we have provided evidence that modulation of expression of CD44s has an impact on the invasion and migration of prostate cancer cells. Little is known about the prognostic value of the expression of variant isoforms of CD44. Expression of CD44v6 may possibly an independent predictor of survival in prostate cancer. As suggested by others [53], our results support the association of expression of CD44v6 with prolonged disease-free with possibly better-survival significance. We show here for the first time that MMP9 knockdown induces noninvasive cellular phenotype in prostate cancer cells.

We have previously demonstrated that CD44s regulates cell survival in PC3 cells [7]. One would expect a decrease in cell viability as CD44s expression is considerably reduced in PC3/Si cells. However, an increase in the expression of CD44v6 independent of MMP9/CD44s regulation compensates this mechanism and supports cell viability. CD44v6 expression inversely correlates with pathologic stage and disease progression and positively correlates with PSA-free survival [54]. We have begun studies designed to determine the specific roles of MMP9 and CD44s in tumor progression. How does this switch "CD44s to CD44v6" in PC3 cells improve survival and what is/are the mechanism(s) tied to this switch needs further elucidation.

CD44 variant isoforms contain new oligosaccharide attachment sites which provide functionally distinct and significant glycosylation changes [55, 56]. Glycosylation of CD44v6 is suppressed in PC3 cells although its expression was observed at transcriptional and translational levels. Differential regulation of CD44v6 seems to occur during normal and nonmalignant state of cells. Glycosylation of CD44v6 may induce the formation of actin stress fibers and focal adhesions. These events can occur only upon the binding of CD44v6 with appropriate ligand.

CD44 variant isoforms v6 and v7 have been shown to confer binding properties with multiple glycosaminoglycans (GAGs) when expressed on the cell surface [57]. CD44 binding with GAGs was suggested to have several functional consequences such as, (a) affinity to ECM to enhance cell-matrix interactions; (b) trigger signal transduction mechanism; (c) present growth factor to its receptor [57]. CD44 variants have also been shown to function as a coreceptor for the activation of growth-promoting tumor receptor tyrosine kinases [58, 59]. As suggested by others, it is possible that variant isoform may either mediate binding to new ligands, or modulate the function of domains expressed on all CD44 proteins, such as the hyaluronic-acid-binding domain. Further understanding of the GAG and ligand binding activities of CD44v6 and its role in the formation of focal adhesions will provide insight into the functions of CD44s and CD44v6 in the metastasis of tumor cells. Further biochemical characterization of how CD44v6 signaling is orchestrated spatiotemporally and complexes of proteins involved in the distinctively different adhesion regimes are in progress. Identification of the ligand that is involved in the signaling events by CD44v6 may provide a clue to develop new strategies to inhibit invasion.

## 5. Conclusions

The spread of cancer cells to distant sites in the body is the major cause of cancer patients' death. PC3 cells knockdown of MMP9 or CD44s fail to degrade the matrix due to the absence of invadopodia. Cells knockdown of these proteins highlights the importance of these proteins in prostate cancer invasion and migration. CD44s induced signaling events are associated with the highly metastatic property of androgen independent prostate cancer cells. PC3/Si (CD44) cells lost their ability to attach to plates due to failure in the formation of focal adhesions. Loss of invasion property in these cells is due to failure in the formation of invadopodia. Glycosylation of CD44v6 may provide a firm adhesive phenotype with the formation of stress fiber and focal adhesions. A switch from CD44 variant isoforms to CD44s can influence the metastatic property of tumor cells. Downregulation of CD44v6 signaling correlates with invasion and metastasis processes via gain of function of CD44s in androgen-independent prostate cancer cells.

## Abbreviations

CD44s: Soluble or Standard CD44
FBS: Fetal bovine serum
GAPDH: Glyceraldehydes-3-phosphate dehydrogenase
RT-PCR: Reverse transcriptase PCR
TMA: Tissue microarray
IP: Immunoprecipitation
IB: Immunoblot
MMP9: Matrix metalloproteinase-9
PC3/Si: PC3 cell lines knockdown of MMP9
TFMSA: Trifluromethanesulphonic acid
CD44v: Variant isoforms of CD44
GAGs: Glycosaminoglycans
BME: Basement membrane extract.

## Conflict of Interests

The author declare no conflict of interests.

## Authors' Contribution

A. Gupta and W. Cao contributed equally to this paper.

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## References

[1] B. Desai, M. J. Rogers, and M. A. Chellaiah, "Mechanisms of osteopontin and CD44 as metastatic principles in prostate cancer cells," *Molecular Cancer*, vol. 6, article 18, 2007.

[2] J. E. Draffin, S. McFarlane, A. Hill, P. G. Johnston, and D. J. J. Waugh, "CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells," *Cancer Research*, vol. 64, no. 16, pp. 5702–5711, 2004.

[3] C. M. Isacke and H. Yarwood, "The hyaluronan receptor, CD44," *International Journal of Biochemistry and Cell Biology*, vol. 34, no. 7, pp. 718–721, 2002.

[4] H. Ponta, J. Sleeman, P. Dall, J. Moll, L. Sherman, and P. Herrlich, "CD44 isoforms in metastatic cancer," *Invasion and Metastasis*, vol. 14, no. 1–6, pp. 82–86, 1994.

[5] D. Naor, R. V. Sionov, M. Zahalka, M. Rochman, B. Holzmann, and D. Ish-Shalom, "Organ-specific requirements for cell adhesion molecules during lymphoma cell dissemination," *Current Topics in Microbiology and Immunology*, vol. 231, pp. 143–166, 1998.

[6] B. Desai, T. Ma, and M. A. Chellaiah, "Invadopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion," *The Journal of Biological Chemistry*, vol. 283, no. 20, pp. 13856–13866, 2008.

[7] B. W. Robertson and M. A. Chellaiah, "Osteopontin induces $\beta$-catenin signaling through activation of Akt in prostate cancer cells," *Experimental Cell Research*, vol. 316, no. 1, pp. 1–11, 2010.

[8] J. Cichy and E. Puré, "The liberation of CD44," *The Journal of Cell Biology*, vol. 161, no. 5, pp. 839–843, 2003.

[9] C. J. N. Rall and A. K. Rustgi, "CD44 isoform expression in primary and metastatic pancreatic adenocarcinoma," *Cancer Research*, vol. 55, no. 9, pp. 1831–1835, 1995.

[10] N. Yamane, S. Tsujitani, M. Makino, M. Maeta, and N. Kaibara, "Soluble CD44 variant 6 as a prognostic indicator in patients with colorectal cancer," *Oncology*, vol. 56, no. 3, pp. 232–238, 1999.

[11] J. W. Stevens, P. L. Palechek, T. L. Griebling, R. J. Midura, O. W. Rokhlin, and M. B. Cohen, "Expression of CD44 isoforms in human tumor cell lines," *Prostate*, vol. 28, pp. 153–161, 1996.

[12] B. Desai, T. Ma, J. Zhu, and M. A. Chellaiah, "Characterization of the expression of variant and standard CD44 in prostate cancer cells: identification of the possible molecular mechanism of CD44/MMP9 complex formation on the cell surface," *Journal of Cellular Biochemistry*, vol. 108, no. 1, pp. 272–284, 2009.

[13] B. L. Lokeshwar, V. B. Lokeshwar, and N. L. Block, "Expression of CD44 in prostate cancer cells: association with cell proliferation and invasive potential," *Anticancer Research*, vol. 15, no. 4, pp. 1191–1198, 1995.

[14] L. Patrawala, T. Calhoun, R. Schneider-Broussard et al., "Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells," *Oncogene*, vol. 25, no. 12, pp. 1696–1708, 2006.

[15] A. C. Gao, W. Lou, J. P. Sleeman, and J. T. Isaacs, "Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate," *Cancer Research*, vol. 58, no. 11, pp. 2350–2352, 1998.

[16] R. G. Pergolizzi, W. Kreis, C. Rottach, M. Susin, and J. D. Broome, "Mutational status of codons 12 and 13 of the N- and K-ras genes in tissue and cell lines derived from primary and metastatic prostate carcinomas," *Cancer Investigation*, vol. 11, no. 1, pp. 25–32, 1993.

[17] O. H. Sanchez-Sweatman, F. W. Orr, and G. Singh, "Human metastatic prostate PC3 cell lines degrade bone using matrix metalloproteinases," *Invasion and Metastasis*, vol. 18, no. 5-6, pp. 297–305, 1998.

[18] A. L. G. Schuurmans, J. Bolt, and E. Mulder, "Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell LNCaP," *Prostate*, vol. 12, no. 1, pp. 55–63, 1988.

[19] C. K. Choo, M. T. Ling, K. W. Chan et al., "Immortalization of human epithelial cells by HPV 16 E6/E7 open reading frames," *Prostate*, vol. 40, pp. 150–158, 1999.

[20] P. Feng, T. L. Li, Z. X. Guan, R. B. Franklin, and L. C. Costello, "Direct effect of zinc on mitochondrial apoptogenesis in prostate cells," *Prostate*, vol. 52, no. 4, pp. 311–318, 2002.

[21] S. W. Hayward, R. Dahiya, G. R. Cunha, J. Bartek, N. Deshpande, and P. Narayan, "Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1," *In Vitro Cellular & Developmental Biology*, vol. 31, no. 1, pp. 14–24, 1995.

[22] S. W. Hayward, Y. Wang, M. Cao et al., "Malignant transformation in a nontumorigenic human prostatic epithelial cell line," *Cancer Research*, vol. 61, no. 22, pp. 8135–8142, 2001.

[23] R. B. Franklin, P. Feng, B. Milon et al., "hZIP1 zinc uptake transporter down regulation and zinc depletion in prostate cancer," *Molecular Cancer*, vol. 4, article 32, 2005.

[24] A. Gupta, W. Cao, and M. A. Chellaiah, "Integrin avb3 and CD44 pathways in metastatic prostate cancer cells support osteoclastogensis via RUNX2/Smad5/RANKL," *Signaling Axis*, vol. 11, pp. 1–18, 2012.

[25] T. Ma, K. Sadashivaiah, and M. A. Chellaiah, "Regulation of sealing ring formation by L-plastin and cortactin in osteoclasts," *The Journal of Biological Chemistry*, vol. 285, no. 39, pp. 29911–29924, 2010.

[26] V. Samanna, H. Wei, D. Ego-Osuala, and M. A. Chellaiah, "Alpha-V-dependent outside-in signaling is required for the regulation of CD44 surface expression, MMP-2 secretion, and cell migration by osteopontin in human melanoma cells," *Experimental Cell Research*, vol. 312, no. 12, pp. 2214–2230, 2006.

[27] M. Chellaiah, N. Kizer, M. Silva, U. Alvarez, D. Kwiatkowski, and K. A. Hruska, "Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength," *The Journal of Cell Biology*, vol. 148, no. 4, pp. 665–678, 2000.

[28] A. Paszkiewicz-Gadek, A. Gindzieński, and H. Porowska, "An improved method for chemical deglycosylation of gastric mucus glycoprotein," *Roczniki Akademii Medycznej w BiaThlymstoku*, vol. 42, no. 1, pp. 18–25, 1997.

[29] A. Schneider, R. H. Younis, and J. S. Gutkind, "Hypoxia-induced energy stress inhibits the mTOR pathway by activating an AMPK/REDD1 signaling axis in head and neck squamous cell carcinoma," *Neoplasia*, vol. 10, no. 11, pp. 1295–1302, 2008.

[30] K. H. Heider, H. Kuthan, G. Stehle, and G. Munzert, "CD44v6: a target for antibody-based cancer therapy," *Cancer Immunology, Immunotherapy*, vol. 53, no. 7, pp. 567–579, 2004.

[31] M. A. Chellaiah, N. Kizer, R. Biswas et al., "Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression," *Molecular Biology of the Cell*, vol. 14, no. 1, pp. 173–189, 2003.

[32] B. W. Robertson, L. Bonsal, and M. A. Chellaiah, "Regulation of Erk1/2 activation by osteopontin in PC3 human prostate cancer cells," *Molecular Cancer*, vol. 9, article 260, 2010.

[33] E. T. Bowden, M. Barth, D. Thomas, R. Glazer, and S. C. Mueller, "An invasion-related complex of cortactin, paxillin and PKC$\mu$ associates with invadopodia at sites of extracellular matrix degradation," *Oncogene*, vol. 18, no. 31, pp. 4440–4449, 1999.

[34] R. Buccione, J. D. Orth, and M. A. McNiven, "Foot and mouth: podosomes, invadopodia and circular dorsal ruffles," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 8, pp. 647–657, 2004.

[35] H. Yamaguchi, F. Pixley, and J. Condeelis, "Invadopodia and podosomes in tumor invasion," *European Journal of Cell Biology*, vol. 85, no. 3-4, pp. 213–218, 2006.

[36] T. Kelly, S. C. Mueller, Y. Yeh, and W. T. Chen, "Invadopodia promote proteolysis of a wide variety of extracellular matrix proteins," *Journal of Cellular Physiology*, vol. 158, no. 2, pp. 299–308, 1994.

[37] G. F. Weber, R. T. Bronson, J. Ilagan, H. Cantor, R. Schmits, and T. W. Mak, "Absence of the CD44 gene prevents sarcoma metastasis," *Cancer Research*, vol. 62, no. 8, pp. 2281–2286, 2002.

[38] V. Orian-Rousseau, "CD44, a therapeutic target for metastasising tumours," *European Journal of Cancer*, vol. 46, no. 7, pp. 1271–1277, 2010.

[39] E. S. Clark, A. S. Whigham, W. G. Yarbrough, and A. M. Weaver, "Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia," *Cancer Research*, vol. 67, no. 9, pp. 4227–4235, 2007.

[40] M. Lorenz, H. Yamaguchi, Y. Wang, R. H. Singer, and J. Condeelis, "Imaging sites of N-WASP activity in lamellipodia and invadopodia of carcinoma cells," *Current Biology*, vol. 14, no. 8, pp. 697–703, 2004.

[41] L. Y. W. Bourguignon, H. Zhu, L. Shao, and Y. W. Chen, "CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration," *The Journal of Biological Chemistry*, vol. 276, no. 10, pp. 7327–7336, 2001.

[42] L. Y. W. Bourguignon, P. A. Singleton, and F. Diedrich, "Hyaluronan-CD44 interaction with Rac1-dependent protein kinase N-$\gamma$ promotes phospholipase C$\gamma$1 activation, Ca2+ signaling, and cortactin-cytoskeleton function leading to keratinocyte adhesion and differentiation," *The Journal of Biological Chemistry*, vol. 279, no. 28, pp. 29654–29669, 2004.

[43] Y. Wang and M. A. McNiven, "Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK-p130Cas complex," *The Journal of Cell Biology*, vol. 196, pp. 375–385, 2012.

[44] U. Gunthert, R. Stauder, B. Mayer, H. J. Terpe, L. Fink, and K. Friedrichs, "Are CD44 variant isoforms involved in human tumour progression?" *Cancer Surveys*, vol. 24, pp. 19–42, 1995.

[45] R. Stauder, W. Eisterer, J. Thaler, and U. Gunthert, "CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor," *Blood*, vol. 85, no. 10, pp. 2885–2899, 1995.

[46] S. Seiter, D. Schadendorf, K. Herrmann et al., "Expression of CD44 variant isoforms in malignant melanoma," *Clinical Cancer Research*, vol. 2, no. 3, pp. 447–456, 1996.

[47] S. Seiter, R. Arch, S. Reber et al., "Prevention of tumor metastasis formation by anti-variant CD44," *Journal of Experimental Medicine*, vol. 177, no. 2, pp. 443–455, 1993.

[48] J. W. R. Mulder, P. M. Kruyt, M. Sewnath et al., "Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins," *The Lancet*, vol. 344, no. 8935, pp. 1470–1472, 1994.

[49] V. J. M. Wielenga, R. van der Voort, J. W. R. Mulder et al., "CD44 splice variants as prognostic markers in colorectal cancer," *Scandinavian Journal of Gastroenterology*, vol. 33, no. 1, pp. 82–87, 1998.

[50] S. B. Fox, J. Fawcett, D. G. Jackson et al., "Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms," *Cancer Research*, vol. 54, no. 16, pp. 4539–4546, 1994.

[51] J. Lesley, R. Hyman, and P. W. Kincade, "CD44 and its interaction with extracellular matrix," *Advances in Immunology*, vol. 54, pp. 271–335, 1993.

[52] D. Naor, R. V. Sionov, and D. Ish-Shalom, "CD44: structure, function, and association with the malignant process," *Advances in Cancer Research*, vol. 71, pp. 241–319, 1997.

[53] K. Friedrichs, F. Franke, B. W. Lisboa et al., "CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer," *Cancer Research*, vol. 55, no. 22, pp. 5424–5433, 1995.

[54] S. Ekici, A. Ayhan, S. Kendi, and H. Özen, "Determination of prognosis in patients with prostate cancer treated with radical prostatectomy: prognostic value of CD44v6 score," *Journal of Urology*, vol. 167, no. 5, pp. 2037–2041, 2002.

[55] K. L. Bennett, B. Modrell, B. Greenfield et al., "Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons," *The Journal of Cell Biology*, vol. 131, no. 6, pp. 1623–1633, 1995.

[56] D. G. Jackson, J. I. Bell, R. Dickinson, J. Timans, J. Shields, and N. Whittle, "Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon," *The Journal of Cell Biology*, vol. 128, no. 4, pp. 673–685, 1995.

[57] J. P. Sleeman, K. Kondo, J. Moll, H. Ponta, and P. Herrlich, "Variant exons v6 and v7 together expand the repertoire of glycosaminoglycans bound by CD44," *The Journal of Biological Chemistry*, vol. 272, no. 50, pp. 31837–31844, 1997.

[58] V. Orian-Rousseau, L. Chen, J. P. Sleeman, P. Herrlich, and H. Ponta, "CD44 is required for two consecutive steps in HGF/c-Met signaling," *Genes and Development*, vol. 16, no. 23, pp. 3074–3086, 2002.

[59] V. Orian-Rousseau, H. Morrison, A. Matzke et al., "Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin," *Molecular Biology of the Cell*, vol. 18, no. 1, pp. 76–83, 2007.

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*Research Article*

# Potentials and Limitations of Real-Time Elastography for Prostate Cancer Detection: A Whole-Mount Step Section Analysis

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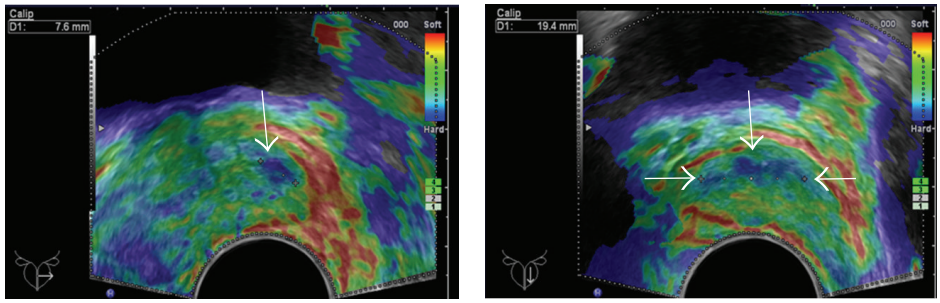
*Objectives*. To evaluate prostate cancer (PCa) detection rates of real-time elastography (RTE) in dependence of tumor size, tumor volume, localization and histological type. *Materials and Methods*. Thirdy-nine patients with biopsy proven PCa underwent RTE before radical prostatectomy (RPE) to assess prostate tissue elasticity, and hard lesions were considered suspicious for PCa. After RPE, the prostates were prepared as whole-mount step sections and were compared with imaging findings for analyzing PCa detection rates. *Results*. RTE detected 6/62 cancer lesions with a maximum diameter of 0–5 mm (9.7%), 10/37 with a maximum diameter of 6–10 mm (27%), 24/34 with a maximum diameter of 11–20 20 mm (70.6%), 14/14 with a maximum diameter of >20 mm (100%) and 40/48 with a volume $\geq 0.2\,cm^3$ (83.3%). Regarding cancer lesions with a volume $\geq 0.2\,cm^3$ there was a significant difference in PCa detection rates between Gleason scores with predominant Gleason pattern 3 compared to those with predominant Gleason pattern 4 or 5 (75% versus 100%; $P = 0.028$). *Conclusions*. RTE is able to detect PCa of significant tumor volume and of predominant Gleason pattern 4 or 5 with high confidence, but is of limited value in the detection of small cancer lesions.

## 1. Introduction

Diagnosis and therapy of prostate cancer (PCa) are discussed controversially. On the one hand, prostate-specific antigen (PSA) testing has low specificity for PCa detection and systematic biopsy low sensitivity, and on the other hand detection of insignificant PCa may lead to overdiagnosis and overtherapy with its cost and complications [1–3]. Strategies like active surveillance, watchful waiting, and focal therapy of index cancer lesions are becoming more popular [3, 4].

Imaging modalities like magnetic resonance imaging (MRI), novel transrectal ultrasound (TRUS) technologies, that is, contrast enhanced TRUS (CE-TRUS), or real-time elastography (RTE) and computer aided analysis of TRUS signals (i.e., HistoScanning or computerized TRUS with artificial neural network analysis) have shown to be helpfully in urological management of diagnosis and/or therapy strategies for PCa [5–8]. One of the key requirements of imaging is to demonstrate significant cancer lesions in the prostate with high confidence, since they may determine the clinical prognosis [4, 9]. Targeted biopsy, focal therapy, and therapy monitoring of these lesions then could become possible. Based on the tumor volume significant lesions are defined to be $\geq 0.2\,cm^3$ or $\geq 0.5\,cm^3$ [10, 11].

One possibility for visualization of PCa is the representation of tissue elasticity. Usually cancers have a higher cell and vessel density than normal tissue and are therefore associated with a decreased elasticity [12, 13]. This is similar to the digital rectal examination (DRE) of the prostate performed by the urologists, where hard palpable areas are classified as




(a) (b)

Figure 1: Hard area PZ mid-gland left measured with 7.6 mm in the axial plane ((a), white arrow) and with 19.4 mm in the sagittal plane ((b), white arrows).

suspicious for PCa. However, only the posterior parts of the prostate can be evaluated by DRE [14]. RTE, an ultrasonic method which is able to demonstrate tissue elasticity color-coded, does not have this problem, since all anatomical regions of the peripheral zone (PZ) can be evaluated [6]. Furthermore, this noninvasive technique is time-and cost-effective and proved its potentials in PCa detection with promising results in former studies [14, 15]. In contrast to static MRI, targeted biopsy and focal therapy of the prostate can be done under real-time conditions with RTE.

The aim of this study was to evaluate PCa detection rates of RTE in dependence of tumor size, tumor volume, localization, and histological type and to determine reasons for false negative findings.

## 2. Materials and Methods

*2.1. Patients.* From April 2010 to November 2011 39 consecutive patients with a median age of 63 years (range: 48–75 years) and a median serum PSA value of 5 ng/mL (range: 2.1–14 ng/mL) participated in this prospective single-center study. All patients were informed about the study design and the study objective. Written informed consent and a positive vote by the local ethics committee of Innsbruck were present. Men with biopsy proven PCa and who were scheduled for radical prostatectomy (RPE) were included. All participants underwent RTE before RPE, and after RPE the prostates were prepared as whole-mount step sections, and the boarder of cancer lesions were marked. DRE was not part of the study.

*2.2. Real-Time Elastography.* RTE was done by one experienced uroradiologist (F. Aigner) on a EUB 8500 Hitachi ultrasound unit (Hitachi medical systems, Tokyo, Japan) using a 7.5 MHz end fire transrectal probe to assess tissue elasticity. Elastograms were obtained by slight prostate compression and decompression, which was manually induced by the investigator using the transrectal probe and controlled by the compression indicator on the monitor. Hard areas were considered PCa suspicious and color coded blue (Figure 1). These areas were reproducible in the axial and sagittal planes using a previously described approach [16]. Imaging findings suspicious for PCa were assigned to anterior, posterior, right, and left parts of the peripheral zone (PZ) of the prostate only, since most cancers originate from this zone and furthermore, transitional zone cancers are more likely to be less aggressive [17, 18].

*2.3. Histopathology: Preparation, Reporting, and Correlation with RTE Findings.* After RPE and fixation, the prostatectomy specimens were laminated in 4 mm thick slices with an orientation of 90° to the urethra and prepared according to the Stanford protocol. Pathological analysis was performed by one dedicated uropathologist (G. Schäfer), who outlined every cancer lesion and reported an assigned Gleason score. Tumor measures were provided in consideration of a shrinkage factor. The whole-mount step sections have been scanned in our system and were used in digital form for a correlation with the data of imaging findings. The PZ was divided in anterior, posterior, right, and left parts and the limit between anterior and posterior part was defined as the section running through the widest transverse diameter of the prostate.

Based on histopathology, the lesions were classified according to their maximal diameter in the following 4 categories: lesions with a maximum diameter of 0–5 mm, 6–10 mm, 11–20 mm, and >20 mm. Furthermore, lesions were classified to their tumor volume in the following 2 categories: lesions with a volume of $\geq 0.2$ cm$^3$ and $\geq 0.5$ cm$^3$.

*2.4. Statistical Analysis.* Cancer detection rates based on tumor size and tumor volume as well as patient characteristics were summarized with frequencies and percentages or with median, range, minimum, and maximum values. The chi-square test was used to calculate significant differences between PCa detection rates based on localization, prostate volume (PV), tumor size, Gleason Scores, and serum PSA values. All statistical calculations were performed using SPSS 18.0 software, and a $P < 0.05$ was considered statistically significant.

## 3. Results

Overall, histological examination of the 39 prostatectomy specimens showed 147 cancer lesions in the PZ with a median size of 7.7 mm (range: 2–30.8 mm) of which 43 (29.3%) were localized in the anterior part, 90 (61.2%) in

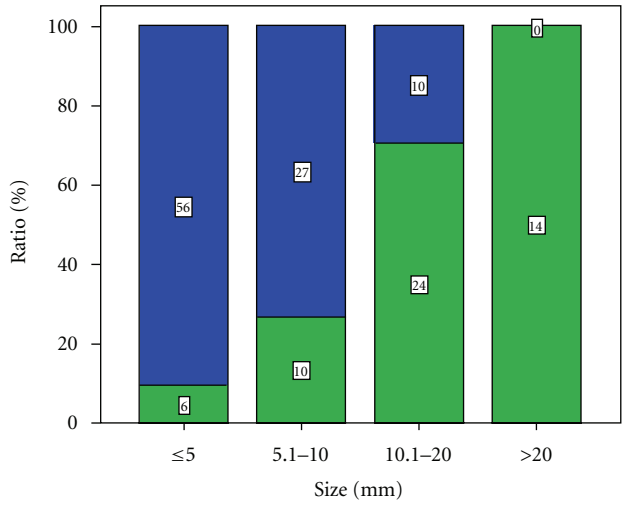



Figure 2: Detection rate based on tumor size.

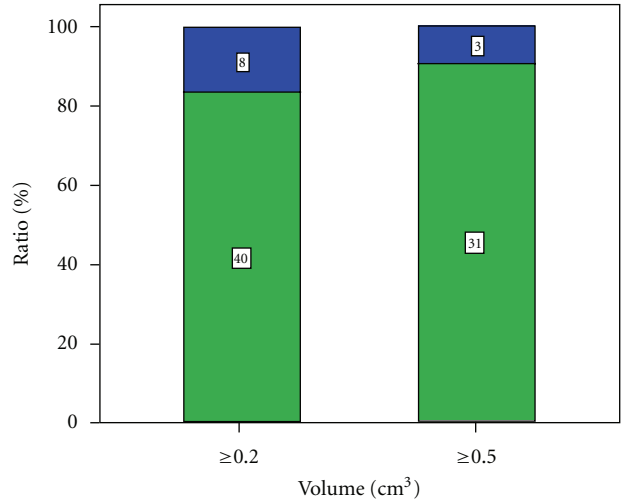



Figure 3: Detection rate based on tumor volume.

the posterior part, and 14 (9.5%) in both anterior and posterior parts of the prostate. RTE detected a total of 54 cancer lesions out of the 147 (36.7%). The median volume when only including tumors ≥0.2 cm$^3$ was 0.85 cm$^3$ (range: 0.21–11.18 cm$^3$). The median Gleason score of all cancer lesions was 6 (range: 5–10) and of cancer lesions ≥0.2 cm$^3$ was 7 (range: 6–10).

*3.1. PCa Detection Rates in Dependence of Tumor Size (Figure 2).* RTE detected 6 of 62 cancer lesions with a maximum diameter of 0–5 mm (9.7%), 10 of 37 with a maximum diameter of 6–10 mm (27%), 24 of 34 with a maximum diameter of 11–20 mm (70.6%) and, 14 of 14 with a maximum diameter of >20 mm (100%).

*3.2. PCa Detection Rates in Dependence of Tumor Volume (Figure 3).* RTE detected 40 of 48 cancer lesions with a tumor volume ≥0.2 cm$^3$ (83.3%) and 31 of 34 with a tumor volume ≥0.5 cm$^3$ (91.2%).

Table 1: Detection rate of all lesions ≥0.2 cm$^3$ in dependence of localization, Gleason scores, prostate volumes and PSA serum values; $n = 48$.

| | Detection | $P$ |
|---|---|---|
| Localization | | |
| Anterior | 66.7% (6/9) | |
| Posterior | 80% (20/25) | 0.419 |
| Both | 100% (14/14) | |
| Gleason score | | |
| G5, G6, G7 (3+4) | 75% (24/32) | 0.028 |
| G7(4+3), G8, G9, G10 | 100% (16/16) | |
| PV (mL) | | |
| <40 | 86.8% (33/38) | 0.204 |
| ≥40 | 70% (7/10) | |
| PSA (ng/mL) | | |
| <4 | 84.6% (11/13) | 0.885 |
| ≥4 | 82.9% (29/35) | |

*3.3. PCa Detection Rates Including all Cancer Lesions ≥0.2 cm$^3$ in Dependence of Localization, Gleason Scores, Prostate Volumes, and PSA Values (Table 1).* RTE detected 6 of 9 cancer lesions in the anterior part (66.7%; group 1), 20 of 25 cancer lesions in the posterior part (80%; group 2), and 14 of 14, if the cancer lesions were located in both anterior and posterior parts (100%; group 3). PCa detection was not significantly different between group 1 and 2 ($P = 0.419$).

There was no significant difference for PCa detection in prostate volumes <40 mL and ≥40 mL ($P = 0.204$) and at serum PSA values <4 ng/mL and ≥4 ng/mL ($P = 0.885$).

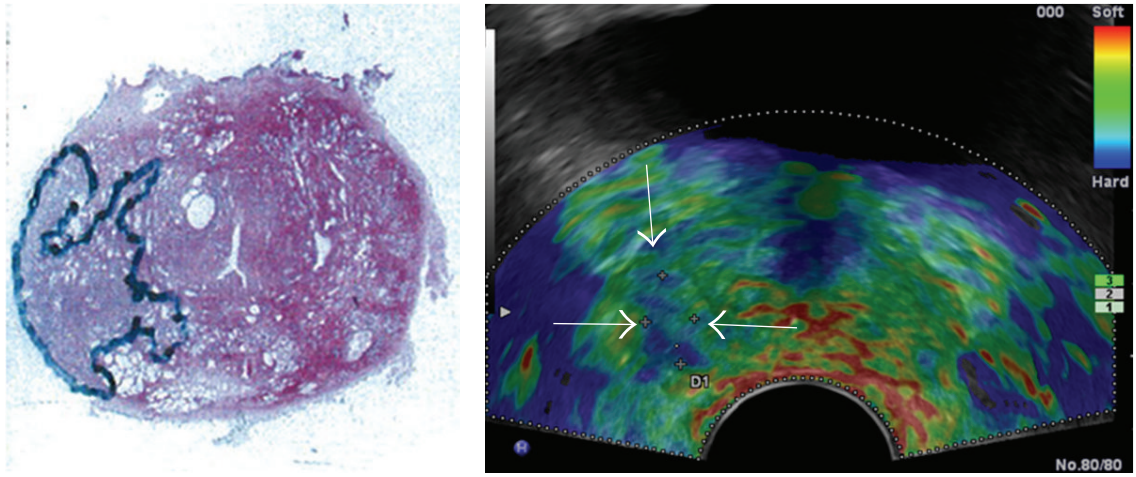
A significant difference in PCa detection was found for PCa with a predominant Gleason pattern ≤3 and ≥4 ($P = 0.028$).

*3.4. False Negative Findings on RTE for Cancer Lesions ≥0.2 cm$^3$.* All 8 missed cancer lesions ≥0.2 cm$^3$ had a predominant Gleason pattern of 3. Six of these eight lesions had sparse architecture on histology, which is despite the malignant components composed of normal glands and glands with dilated lumina. Only two lesions were dense and had tumor volumes of 0.41 cm$^3$ and 0.22 cm$^3$, respectively.

## 4. Discussion

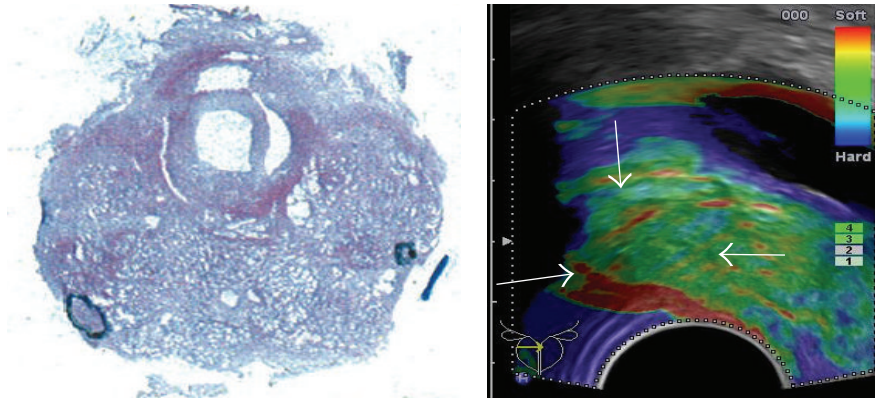
A total of 26% (10/39) of our study population had serum PSA values <4 ng/mL at time of biopsy and have been cancer positive. This suggests that there is no sufficient PSA cutoff which allows excluding PCa. Thompson et al. and our study group demonstrated this fact in former studies [19, 20].

Therefore, imaging modalities to visualize PCa should raise the confidence for PCa detection independently of serum PSA values and should demonstrate significant cancer disease with high sensitivities. Detecting insignificant disease may lead to overdiagnosis and overtherapy [3]. Some authors define cancer lesions <0.5 cm$^3$ as insignificant, whereas other prefer a treshold of <0.2 cm$^3$ [10, 11]. In our series, RTE was capable to demonstrate 83.3% of all cancer lesions with




(a) (b)

Figure 4: Outlined large cancer lesion PZ midgland right on whole-mount step section shown on (a) and corresponding hard area (white arrows) on elastogram (b) in axial planes.




(a) (b)

Figure 5: Outlined small cancer lesion PZ base right on whole-mount step section shown on (a) and corresponding elastogram on (b) in axial plane with arrow marked soft base.

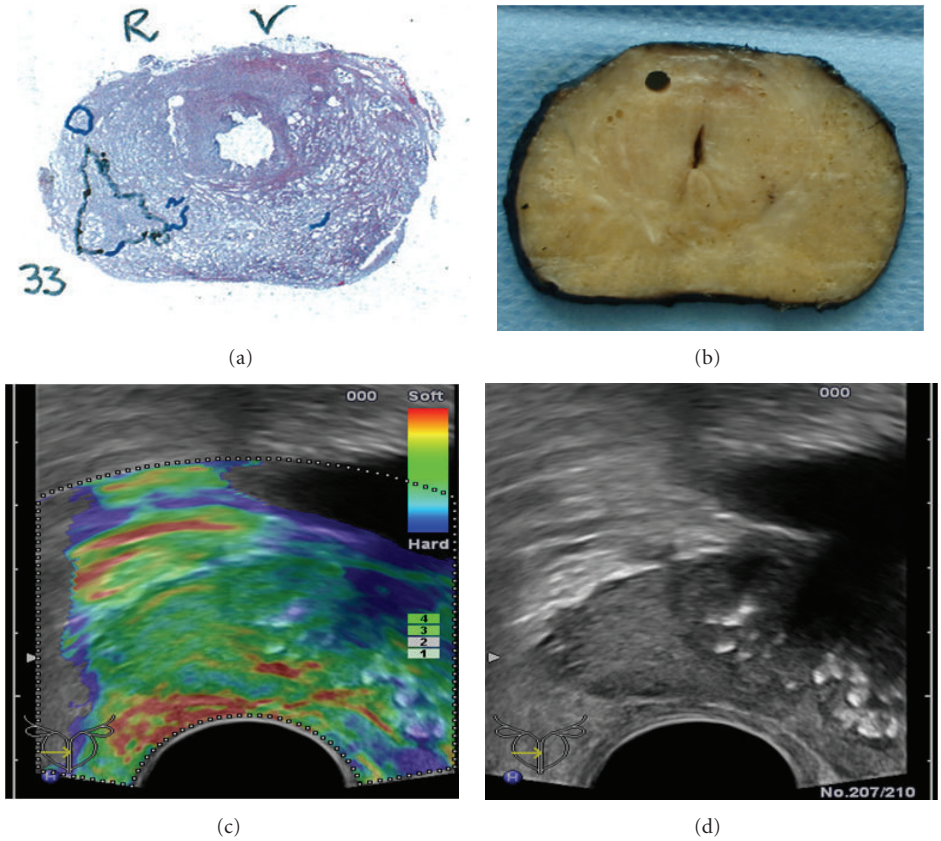
a tumor volume $\geq 0.2\,cm^3$ and 91.2% with a tumor volume of $\geq 0.5\,cm^3$ (Figure 4).

Regarding the largest diameter the detection rate in the group 0–5 mm was weak with 9.7%, also not satisfying in the group 6–10 mm with 27% (Figure 5). However, as stated above: should we really be able to detect those small cancer lesions?

Roethke et al. investigated tumor size dependent detection rates of well-established T2 weighted magnetic resonance imaging (T2w-MRI) and found sensitivities of 45% and 89% for lesions with a maximum size of 10–20 mm and >20 mm, which is slightly lower than our results (70.6% and 100%; resp.) [21]. Furthermore, they concluded that T2w-MRI cannot exclude PCa with lesions smaller 10 mm and $0.4\,cm^3$ and that including foci smaller 10 mm or less than $0.5\,cm^3$ decreased sensitivity clearly. Similar to our results, the presented data suggest that generally imaging of PCa is limited due to tumor size. Nevertheless, they considered their detection rate for lesions more than 20 mm ($1.6\,cm^3$) as high [21].

In contrast, Walz et al. concluded that RTE alone did not allow the identification of the PCa index lesion with satisfactory reliability, which should be necessary for focal therapy. They compared RTE findings with whole-mount step sections to evaluate the diagnostic accuracy for identifying the PCa index lesion, which is considered to be responsible for possible metastatic progression and cancer-specific death and observed a sensitivity of only 58.8% [4].

Sumura et al. reported sensitivities for RTE of 72.7% for tumors with volume $<0.1\,cm^3$, 77.8% for tumors with volume 0.1–0.3 $cm^3$, 71.4% for tumors with volume $>0.3\,cm^3$, and 100% for tumors with volume $>0.5\,cm^3$ [22]. Similar to our study, the detection rates for both anterior and posterior tumors were nearly equal. Furthermore, our data indicate that PV and PSA serum values have no significant influence for detection rates in significant disease (Table 1).




(a) (b)

(c) (d)

Figure 6: Outlined sparse cancer lesion PZ base right on whole-mount step section (a), no suspicious changes on macroscopic specimen (b), elastogram (c), and grey-scale ultrasound (d).

Nevertheless, we missed 8 of 48 cancer lesions with a tumor volume >0.2 $cm^3$ on RTE, which means nearly 20% of significant disease. Our pathologist reevaluated the whole-mount step sections of these 8 cases and all missed cancer lesions had a predominant Gleason pattern 3 and no cancer lesion with predominant Gleason pattern of 4 or 5 was missed.

In total, 6 of the 8 cancer lesions showed sparse architecture on histology, which means they were intermixed with normal glands and also with glands showing dilated lumina and so are more fluid and therefore soft which is a limitation for RTE (Figure 6). The 2 other cases were dense tumors and had volumes of 0.22 $cm^3$ and 0.41 $cm^3$. This fact was also described by Langer et al. who investigated the outcome of diffusion weighted MRI (DWI) and T2w-MRI in dependence of histological tumor composition [23]. Similar to RTE, DWI assesses tissue information due to cell density: the denser the cancer, the higher the diffusion restriction. All of their "invisible" tumors also had predominant Gleason pattern 3 and showed sparse architecture on histology, so that they did not significantly differ from healthy prostate tissue. The authors concluded that this may limit T2w-MRI and DWI in the detection and the assessment of tumor volume of some cancers.

Some cancer lesions may be negative on RTE, but positive on CE-TRUS [20]. Maybe this multiparametric way—adding tissue informations about contrast media dynamics to grey-scale ultrasound and RTE—could have detected some of our false negative findings. Brock et al. have shown the usefulness of this approach, whereas Nygård et al. demonstrated the benefit of adding new biomarkers, like PCA-3, to RTE findings for the detection of significant disease [24, 25].

Our study has several limitations. (1) We do not have data about intra- and interobserver variability. (2) We have used only one US system for RTE. The reproducibility of our results with other US systems needs to be evaluated in further studies. (3) We focused this study on correlating tumor sizes; we did not correlate right negative results between RTE and histopathologic specimens. (4) We investigated the PZ only due to the above mentioned reasons. (5) We knew that every patient had PCa, which is a bias. (6) The planes of whole-mount step sections had an orientation of 90° to the urethra, while an endfire transducer provides images in different angles. Therefore it could be difficult to be sure, whether the identical geographic areas were compared. An investigation with 3D/4D ultrasound would be desirable.

## 5. Conclusion

RTE is capable of detecting significant PCa with high sensitivity, but can have problems when visualizing tumors with sparse architecture. Therefore, adding information about

contrast media dynamics in a multiparametric way may decrease the number of false negative cases. In the detection of smaller cancer lesions, RTE is of limited value.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

D. Junker, M.D., and G. Schäfer, M.D., are equally contributing authors.

## References


[1] F. Gómez Veiga, J. Ponce Reixa, A. Barbagelata López, and M. González Martín, "Current role of PSA and other markers in the diagnosis of prostate cancer," *Archivos Espanoles de Urologia*, vol. 59, no. 10, pp. 1069–1082, 2006.

[2] M. Norberg, L. Egevad, L. Holmberg, P. Sparén, B. J. Norlén, and C. Busch, "The sextant protocol for ultrasound-guided core biopsies of the prostate underestimates the presence of cancer," *Urology*, vol. 50, no. 4, pp. 562–566, 1997.

[3] A. Hegele, L. Skrobek, R. Hofmann, and P. Olbert, "Multiparametric MRI, elastography, contrastenhanced TRUS. Are there indications with reliable diagnostic advantages before prostate biopsy?" *Urologe A*, vol. 51, no. 9, pp. 1270–1277, 2012.

[4] J. Walz, M. Marcy, J. T. Pianna, S. Brunelle, G. Gravis, and N. Salem, "Identification of the prostate cancer index lesion by real-time elastography: considerations for focal therapy of prostate cancer," *World Journal of Urology*, vol. 29, no. 5, pp. 589–594, 2011.

[5] J. Kurhanewicz, D. Vigneron, P. Carroll, and F. Coakley, "Multiparametric magnetic resonance imaging in prostate cancer: present and future," *Current Opinion in Urology*, vol. 18, no. 1, pp. 71–77, 2008.

[6] F. Aigner, G. Schäfer, E. Steiner et al., "Value of enhanced transrectal ultrasound targeted biopsy for prostate cancer diagnosis: a retrospective data analysis," *World Journal of Urology*, vol. 30, no. 3, pp. 341–346, 2012.

[7] B. Grabski, L. Baeurle, A. Loch, B. Wefer, U. Paul, and T. Loch, "Computerized transrectal ultrasound of the prostate in a multicenter setup (C-TRUS-MS): detection of cancer after multiple negative systematic random and in primary biopsies," *World Journal of Urology*, vol. 29, no. 5, pp. 573–579, 2011.

[8] L. A. M. Simmons, P. Autier, F. Zát'ura et al., "Detection, localisation and characterisation of prostate cancer by prostate HistoScanning," *British Journal of Urology International*, vol. 110, no. 1, pp. 28–35, 2012.

[9] H. U. Ahmed, "The index lesion and the origin of prostate cancer," *New England Journal of Medicine*, vol. 361, no. 17, pp. 1704–1706, 2009.

[10] N. B. Delongchamps, M. Rouanne, T. Flam et al., "Multiparametric magnetic resonance imaging for the detection and localization of prostate cancer: combination of T2-weighted, dynamic contrast-enhanced and diffusion-weighted imaging," *British Journal of Urology International*, vol. 107, no. 9, pp. 1411–1418, 2011.

[11] A. M. Wise, T. A. Stamey, J. E. McNeal, and J. L. Clayton, "Morphologic and clinical significance of multifocal prostate cancers in radical prostatectomy specimens," *Urology*, vol. 60, no. 2, pp. 264–269, 2002.

[12] S. A. Bigler, R. E. Deering, and M. K. Brawer, "Comparison of microscopic vascularity in benign and malignant prostate tissue," *Human Pathology*, vol. 24, no. 2, pp. 220–226, 1993.

[13] T. A. Krouskop, T. M. Wheeler, F. Kallel, B. S. Garra, and T. Hall, "Elastic moduli of breast and prostate tissues under compression," *Ultrasonic Imaging*, vol. 20, no. 4, pp. 260–274, 1998.

[14] M. Brock, C. Von Bodman, R. J. Palisaar et al., "The impact of real-time elastography guiding a systematic prostate biopsy to improve cancer detection rate: a prospective study of 353 patients," *Journal of Urology*, vol. 187, no. 6, pp. 2039–2043, 2012.

[15] J. Walz, M. Marcy, T. Maubon et al., "Real time elastography in the diagnosis of prostate cancer: comparison of preoperative imaging and histology after radical prostatectomy," *Progres en Urologie*, vol. 21, no. 13, pp. 925–931, 2011.

[16] K. König, U. Scheipers, A. Pesavento, A. Lorenz, H. Ermert, and T. Senge, "Initial experiences with real-time elastography guided biopsies of the prostate," *Journal of Urology*, vol. 174, no. 1, pp. 115–117, 2005.

[17] A. E. Pelzer, J. Bektic, A. P. Berger et al., "Are transition zone biopsies still necessary to improve prostate cancer detection? Results from the tyrol screening project," *European Urology*, vol. 48, no. 6, pp. 916–921, 2005.

[18] D. R. Greene, T. M. Wheeler, S. Egawa, J. K. Dunn, and P. T. Scardino, "A comparison of the morphological features of cancer arising in the transition zone and in the peripheral zone of the prostate," *Journal of Urology*, vol. 146, no. 4, pp. 1069–1076, 1991.

[19] I. M. Thompson, D. K. Pauler, P. J. Goodman et al., "Prevalence of prostate cancer among men with a prostate-specific antigen level ≤4.0 ng per milliliter," *New England Journal of Medicine*, vol. 350, no. 22, pp. 2239–2246, 2004.

[20] F. Aigner, L. Pallwein, D. Junker et al., "Value of real-time elastography targeted biopsy for prostate cancer detection in men with prostate specific antigen 1.25 ng/ml or greater and 4.00 ng/ml or less," *Journal of Urology*, vol. 184, no. 3, pp. 913–917, 2010.

[21] M. C. Roethke, M. P. Lichy, L. Jurgschat et al., "Tumorsize dependent detection rate of endorectal MRI of prostate cancer—a histopathologic correlation with whole-mount sections in 70 patients with prostate cancer," *European Journal of Radiology*, vol. 79, no. 2, pp. 189–195, 2011.

[22] M. Sumura, K. Shigeno, T. Hyuga, T. Yoneda, H. Shiina, and M. Igawa, "Initial evaluation of prostate cancer with real-time elastography based on step-section pathologic analysis after radical prostatectomy: a preliminary study," *International Journal of Urology*, vol. 14, no. 9, pp. 811–816, 2007.

[23] D. L. Langer, T. H. Van Der Kwast, A. J. Evans et al., "Intermixed normal tissue within prostate cancer: effect on MR imaging measurements of apparent diffusion coefficient and T2-sparse versus dense cancers," *Radiology*, vol. 249, no. 3, pp. 900–908, 2008.

[24] M. Brock, T. Eggert, R. J. Palisaar et al., "Multiparametric ultrasound of the prostate: adding contrast enhanced ultrasound to real-time elastography to detect histopathologically confirmed cancer," *Journal of Urology*, vol. 189, no. 1, pp. 93–98, 2013.

[25] Y. Nygård, S. A. Haukaas, and J. E. Waage, "Combination of real-time elastography and urine prostate cancer gene 3 (PCA3) detects more than 97% of significant prostate cancers," *Scandinavian Journal of Urology and Nephrology*. In press.